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# TUBERCULIN SENSITIVITY OF SENSITIZED LYMPHOCYTES

By

S. TUBOLY, T. SZÁNTÓ and Marietta RÁDY

Central Veterinary Institute (Director: A. VÁNYI), Budapest

(Received August 2, 1972)

The immunology of tuberculosis and the related allergic condition were extensively studied during the last decades (BARAM and MOSKO, 1962; BLOOM and CHAUSE, 1967; KANAI, 1967; LAWRENCE, 1955; MACKNESS and BLANDEN, 1967; OLIVERA-LIMA, 1958). These investigations disclosed much new information, but the findings were often contradictory and numerous problems are still unclear.

The present studies were initiated to determine the sensitivity to tuberculoproteins of lymphocytes from guinea pigs infected with various *Mycobacterium* species and to observe the degrees of blast transformation induced by various mycobacterial antigens.

The applied method was based on the blast transformation of lymphocytes. OPPENHEIM (1968) interpreted blast transformation as the development of small lymphocytes in to lymphoblast cells, whereas LING (1964) saw in it a sign of antibody production, interpreting it as a secondary *in vitro* response to an antigen which the host had already encountered *in vivo*. COWLING *et al.* (1963) and MILLS (1966) studying the response to tuberculin of lymphocyte cultures obtained from tuberculin (Mantoux) found blast transformation, in 20 and 5% of the lymphocytes respectively positive and negative individuals.

Other investigators (FARLAND and HEILMAN, 1966; GERALD, 1968; PESRMIN *et al.*, 1962; SCHRECK, 1963) established in similar studies a correlation between the positive transformation test and tuberculin positivity. HIRSCHORN (1968) found that the *in vitro* immune response reaches maximum on the 5th day after addition of the antigen and that the usual percentage of transformation is 15—20%, but it may reach even 45%.

## Materials and Methods

Sensitization of guinea pigs. Animals were divided into five groups and were infected with the following *Mycobacterium* strains:

1. *Mycobacterium bovis* BCG Budapest
2. *Mycobacterium avium* Chester 15769 (American Type Culture Collection)
3. *Mycobacterium ulcerans* 19423 (ATCC)
4. *Mycobacterium xenopei* Budapest, Korányi Institute for Tuberculosis
5. Uninfected control group

Six-week cultures grown in Lowenstein—Jensen medium were used for experimental infection. A dose of 0.5 ml was administered intraperitoneally

throughout, using an 0.01 mg/ml suspension from *M. bovis* and 0.1 mg/ml suspensions from the other cultures, as calculated for wet weight.

*Sampling and collection of white blood cells.* In each group, individual heparinized blood samples were withdrawn every week for 8 weeks. The samples were incubated for 2 hours at 37 °C, in tubes inclined at an angle of 30°. The white blood cells were then withdrawn in a capillary pipette, passed through glass cotton and nylon mesh, and suspended in Parker's 199 medium.

*Antigens.* The white blood cell cultures were challenged with three antigens: Bovis PPD, Avium PPD and an antigen designated Ps, which had been prepared from the bovis strain $An_5$ by electrophoresis and column chromatography. The challenge dose was 250 $\mu$g/ml cell culture.

Samples were taken on 8 occasions at 24-hour intervals after addition of the antigen. Blast cells were counted in preparations stained with May-Grünwald and Giemsa solutions.

## Results

The blast transformation-inducing activity of the three antigens determined with lymphocytes from each guinea pig group. The results are shown in Fig. 1.

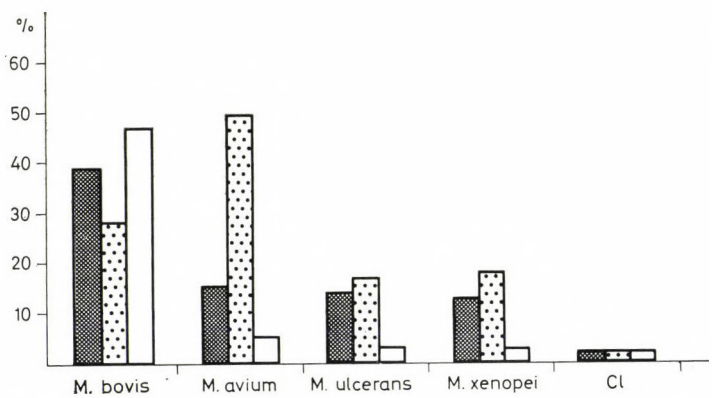


*Fig. 1.* The transformation values of lymphocytes sensitized with *M. bovis*, *M. avium*, *M. ulcerans* or *M. xenopei*. ■ B. PPD; ▒ A. PPD; □ Ps ant.

The variance analysis of numerical results is shown in Table I. Both Bovis PPD and Avium PPD induced blast transformation in lymphocytes of all infected guinea pigs, but the percentage of transformation differed with the strain used. Both antigens displayed maximum activity in the homologous system, being much less active in cell cultures from guinea pigs infected with *M. ulcerans* or *M. xenopei*.

**Table I**

Biometric analysis of lymphocyte sensitivity
Blast transformation per cent

| Groups of guinea pigs infected with | Antigen | Lower limit value | Mean | Upper limit value | SEM* | Var. coeff. | Fidencial limits | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | lower | upper |
| | B. PPD | 24 | 39.1 | 57 | 10.4 | 26.6 | 31.8 | 46.4 |
| *M. bovis* | A. PPD | 20 | 28.3 | 32 | 3.7 | 13.7 | 25.8 | 30.7 |
| | Ps | 36 | 46.9 | 64 | 8.3 | 17.7 | 39.1 | 52.7 |
| | B. PPD | 5 | 15.6 | 23 | 4.7 | 30.6 | 12.6 | 18.9 |
| *M. avium* | A. PPD | 29 | 49.3 | 58 | 8.4 | 17.0 | 43.3 | 55.3 |
| | Ps | 2 | 5.3 | 13 | 3.0 | 57.0 | 3.3 | 7.3 |
| | B. PPD | 8 | 13.7 | 20 | 3.1 | 23.2 | 11.4 | 15.9 |
| *M. ulcerans* | A. PPD | 12 | 16.7 | 26 | 3.9 | 23.4 | 14.0 | 19.4 |
| | Ps | 1 | 2.5 | 5 | 1.4 | 56.0 | 1.6 | 3.4 |
| | B. PPD | 10 | 12.9 | 16 | 2.0 | 16.0 | 11.4 | 14.3 |
| *M. xenopei* | A. PPD | 14 | 17.7 | 20 | 2.1 | 11.8 | 16.3 | 19.1 |
| | Ps | 1 | 2.9 | 5 | 1.2 | 43.7 | 2.0 | 3.8 |
| | B. PPD | 0 | 2.0 | 4 | 1.1 | 55.0 | 1.2 | 2.7 |
| Non infected control | A. PPD | 0 | 1.8 | 4 | 1.1 | 62.0 | 1.0 | 2.5 |
| | Ps | 0 | 1.6 | 3 | 0.9 | 60.0 | 0.9 | 2.2 |

* SEM, standard error of the mean

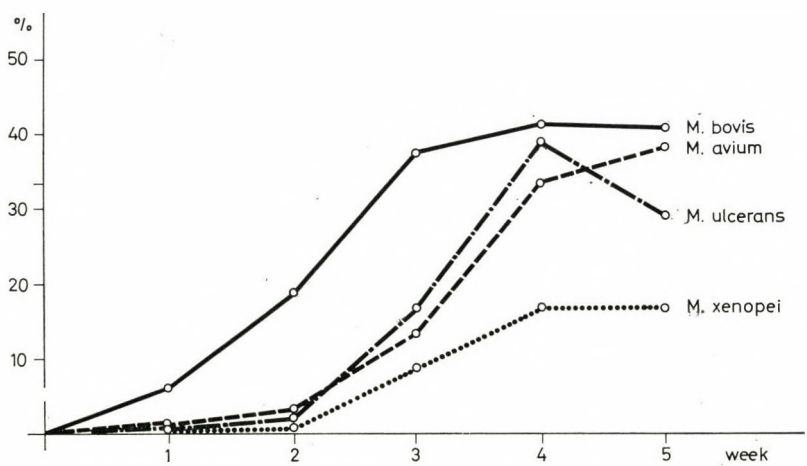


*Fig. 2.* The blast transformation activity of three antigens determined with lymphocytes

The *M. bovis* antigen Ps was more active in the homologous system than Bovis PPD, but failed to induce blast transformation in lymphocytes from guinea pigs infected with the other strains.

Transformed cells are shown in Figs 3 and 4. The dynamics of the development of sensitivity varied with the sensitiving strains. With *M. bovis*, maximum transformation rate (40% with homologous antigen) was reached 3 weeks after the infection. The lymphocytes from guinea pigs sensitized with *M. avium*, *M. ulcerans* or *M. xenopei* showed maximum transformation values from the 4th week on (Fig. 2).

Part of the lymphoblasts extended thread-like processes, variable in length, from the cell surface between the 48 and 72 hours of culturing (Fig. 5).

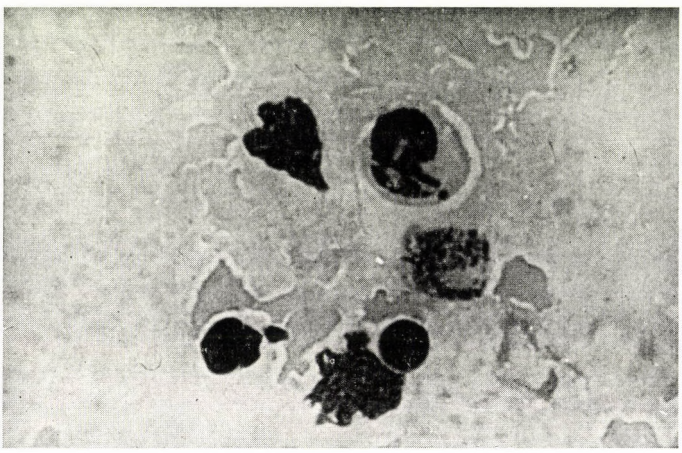

*Fig. 3.* Transformed lymphoblast

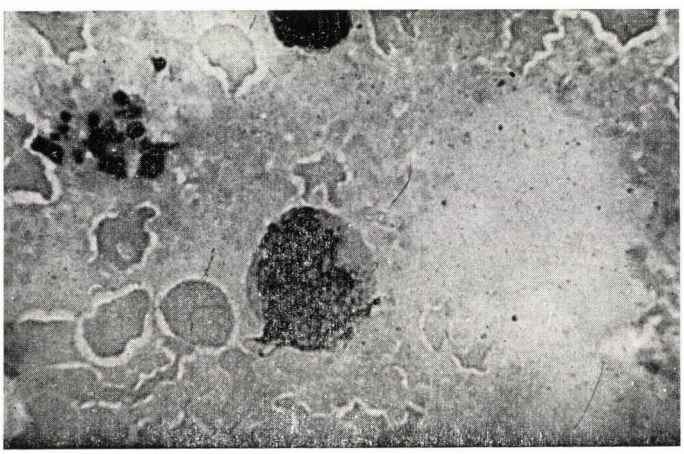

*Fig. 4.* Transformed lymphoblast

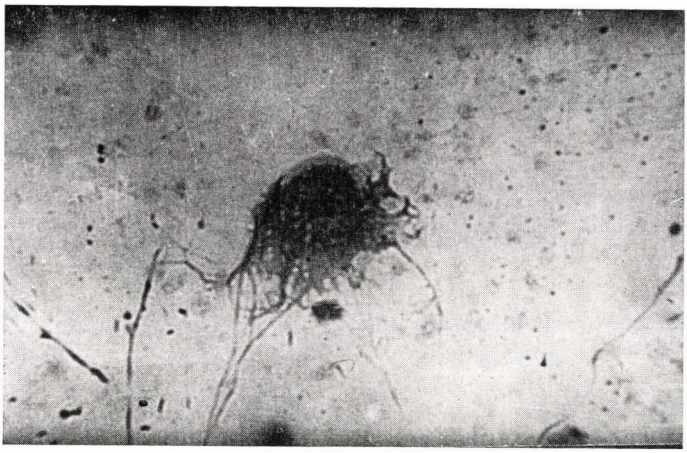

*Fig. 5.* Lymphoblast with thread-like processes

Threads were seen either along the entire circumference of the cell, or occurred only over a minor area of the cell surface. Such forms covered 5—8% of the transformed cells in homologous systems.

## Discussion

The sensitivity of lymphocytes from guinea pigs infected with various *Mycobacterium* species was challenged with three different *Mycobacterium* antigens.

The antigens Bovis PPD and Avium PPD induced transformation of lymphocytes from all infected animals. For lymphocytes of guinea pigs sensitized with *M. bovis*, the transformation rate with the two antigens was approximately the same, but considerably differed in systems sensitized with *M. avium*. The lymphocytes cultures prepared from animals sensitized with *M. ulcerans*, or *M. xenopei* the two antigens appeared to be approximately equally active.

The antigen Ps, prepared from the $AN_5$ strain of *M. bovis*, induced transformation exclusively in *M. bovis*-sensitized lymphocytes, but in these to a greater degree than Bovis PPD. It had no influence whatever on lymphocytes from guinea pigs sensitized with strains other than *M. bovis*.

Thread-like structures like those observed by us on the surface of lymphoblasts between the 48th and 72nd hours of culturing had been described by THEIN and BUSCHMANN (1970), who attributed them to an antigen–antibody reaction. Although the phenomenon occurred regularly in our experiments, we are of the opinion that the liability of lymphocytes to injury must not be disregarded as one of its possible causes.

## Summary

Lymphocytes from guinea pigs infected with *Mycobacterium bovis*, *M. avium*, *M. ulcerans* or *M. xenopei* were tested for sensitivity to the mycobacterium antigens Bovis PPD, Avium PPD and a purified *M. bovis* antigen designated Ps. The blast transformation-inducing activity of each antigen was determined.

Bovis PPD and Avium PPD induced blast transformation in cultures sensitized with *M. bovis* in an approximately equal percentage whereas they considerably differed in activity as tested in *M. avium*-sensitized systems. They showed again, nearly equal activity in *M. ulcerans* and *M. xenopei* systems.

The antigen Ps, which had been prepared from the $AN_5$ strain of *M. bovis*, induced blast transformation exclusively in the homologous system.

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The address of the authors: Dr. Sándor Tuboly, dr. Tibor Szántó, dr. Marietta Rády, 1581 Budapest, Tábornok u. 2, Hungary.

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# EPIZOOTOLOGICAL AND CLINICAL STUDY OF FELINE PANLEUCOPENIA

## (INFECTIOUS GASTROENTERITIS OF THE CAT)

By

Z. HORVÁTH, L. PAPP and A. BARTHA

Department of Internal Diseases (Head: Z. HORVÁTH) and Institute of Epizootology and Bacteriology (Head: J. MÉSZÁROS), University of Veterinary Science, Budapest

(Received November 24, 1972)

As for several years a substantial part of the cats examined in the Small Animal Hospital of the Department of Internal Diseases suffered from viral panleucopenia (VP), an opportunity presented for detailed investigations into the epizootology and clinical aspects of this disease.

Attempts was made to establish the seasonal fluctuation and age group distribution of the disease, to differentiate its clinical forms and to develop effective therapeutic measures. Virus strains were isolated and an experimental vaccine was prepared for preventive purposes.

Infectious feline gastroenteritis was first described by VERGE and CHRISOFORONI in 1928 (cit. MANNINGER and MÓCSY, 1965). In the meantime the disease was recognized all over the world and numerous authors contributed new data to the knowledge of its epizootology and symptomatology) ACKERMANN, 1959; CHRISTOPH and VIERNEISEL, 1961; DAVIDSON and STOCKING, 1952; FINDLAY, 1932; FREUDIGER, 1971; KRAFT, 1961; MEHLS, 1950; SCHULZE, 1954; VOGEL, 1961; WEYERS, 1954), The disease was described under various names, referring to one or another characteristic pathological, haematological, epizootological or clinical feature of the condition. Some of these terms were entirely incorrect and misleading, such as "feline distemper", "feline typhoid", "feline plague", "pseudo-membranaceous enteritis". Recently the names "infectious agranulocytosis", "infectious enteritis" and "infectious gastroenteritis" have been preferred.

KREMBS and SIEGFRIED (1936), and later also others (ENGLERT, 1953; LINDAUER, 1953), described a VP-like cat disease, which, in their opinion, was aetiologically distinct from VP, and termed it infectious laryngo-enteritis. In the meantime several authors (CHRISTOPH, 1963; KIRK, 1971; RÖHRER, 1968; WILKINSON, 1966) have advocated the aetiological identity of the two conditions, but MANNINGER and MÓCSY (1965) described infectious feline laryngoenteritis as an independent disease in their textbook.

The introduction of the tissue culture technique into virological research made the isolation of the causal agent of VP possible. JOHNSON (1964, 1965) identified the virus isolated from a Zoo leopard that had succumbed with gastroenteritis as the aetiological agent of the panleucopenia and gastroenteritis, occasionally causing outbreaks in cats. This disease proved to be aetiologically and clinically identical with the infectious panleucopenia of the domestic cat.

In Hungary, up to now only JÁRMAI (1934) has reported studies on VP of the domestic cat. Recently VETÉSI and BALSAI (1971) observed VP in carcases of racoons kept in the Budapest Zoo.

## Material and Methods

On the basis of ten-year statistics (1962—1971) of the Small Animal Hospital, the absolute number and percentage of cats admitted with VP were established (Table I) and a curve was constructed for seasonal and annual

**Table I**

Incidence of viral panleucopenia among the cats admitted to the Small Animal Hospital (University of Veterinary Science) in the period from 1962 to 1971

| Year | 1962 | 1963 | 1964 | 1965 | 1966 | 1967 | 1968 | 1969 | 1970 | 1971 | Totals |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Total no. of cat patients | 603 | 745 | 704 | 828 | 849 | 856 | 785 | 816 | 875 | 883 | 7944 |
| Cats suffering from VP | 137 | 163 | 154 | 231 | 195 | 194 | 206 | 138 | 167 | 185 | 1770 |
| Percentage | 22.7 | 21.8 | 21.8 | 27.9 | 22.9 | 22.6 | 26.3 | 16.9 | 19.0 | 20.9 | 22.3 |

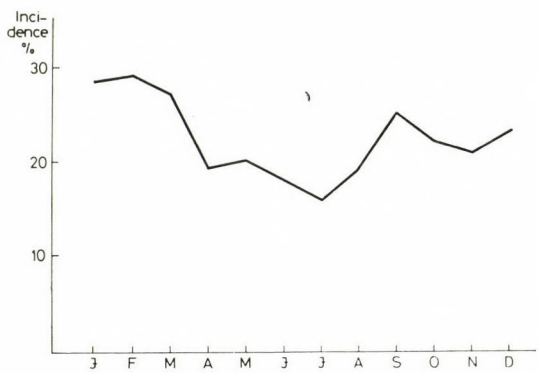


*Fig. 1.* Seasonal fluctuation of viral panleucopenia, 1962—1971

fluctuations of incidence (Fig. 1). Further, cats suffering from VP were grouped according to age (age groups: 2—3, 4—6, 7—12 months, 1.5—2, 3, 4, 5, 6 and 7—8 years) and the age group distribution of incidence and percentual losses was plotted out graphically (Fig. 2).

Diagnosis was established on the basis of characteristic symptoms and haematological findings. Diagnostic data were then compared with the post-mortem findings recorded in the Department of Pathological Anatomy.

Isolation of the causal agent was attempted from various organs (pharynx, lungs, small intestine, spleen, liver) of 47 cats died with symptoms of VP, using cat renal epithelial cell cultures. Secondary cell cultures were more suitable for virus isolation than primary ones.

The virus was isolated from various organs of cats Nos 463, 544, 416, 553, 423 and 537. In such cases the virus was recovered from all examined organs. The virus proved to be chloroform resistant, and specific antisera neutralized it up to a titre of 1 : 32.

Four healthy, susceptible cats aged 3—4 months were infected intranasally and orally with the fourth passage of an isolate. Two cats of similar body weight served as controls. Clinical examinations were performed daily

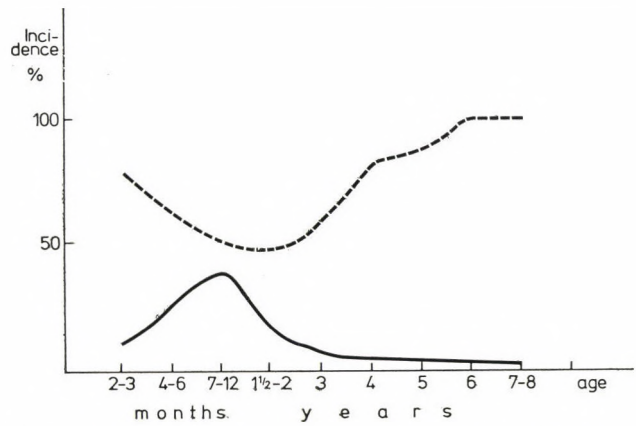


*Fig. 2.* Incidence (———) of, and losses (— · — · —) from viral panleucopenia in the different age groups

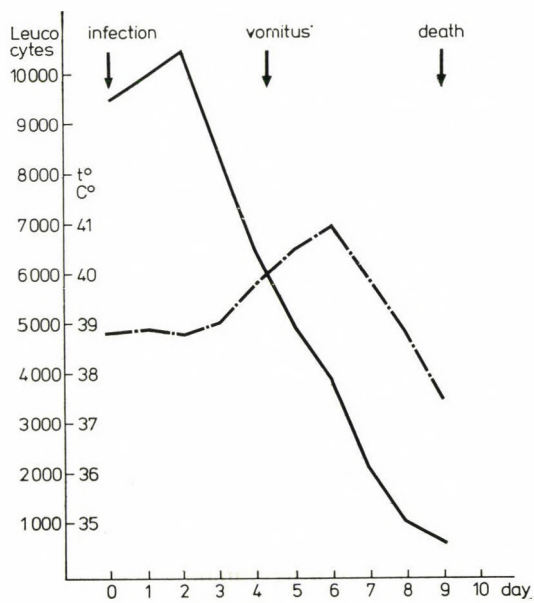


*Fig. 3.* Body temperature (— · — · — ·) and leucocyte count of experimentally infected cat No. 271/a

after the experimental infection and erythrocyte and leucocyte counts as well as differential counts were determined every other day. A detailed urine analysis was also carried out. The characteristic symptoms of VP began to appear 4—5 days after infection. Body temperature values and leucocyte counts of cat No. 271/a, from experimental infection to death, are shown in Fig. 3. The virus could be reisolated from the carcases of two cats which died 9 and 10 days after infection. The control animals were free from symptoms throughout. In another infection experiment, also performed on 4 cats, one animal died; the controls again remained healthy. Convalescent sera were found to contain specific antibodies which neutralized exclusively the virus of VP.

## Preparation and trial of vaccine

The virus isolated from cat No. 537 was propagated in secondary cat epithelial cell culture for 6 days, then 0.2% concentrated formaldehyde solution was added and the formalin-treated virus was allowed to stand at room temperature for 3 days.

The vaccine was tested for immunogenicity in infection experiments. The cats were examined for health status, erythrocyte, leucocyte and differential counts were determined and the usual urine analysis was performed. Three cats aged 4—5 months were each given subcutaneously one ml of the experimental vaccine; another three cats of similar age were treated with the inactivated organ suspension Felidovac (Behringwerke) by the same route. Virus-neutralizing antibodies appeared in the sera of the cats after 14 days. The titres ranged from 1 : 16 to 1 : 128. The six vaccinated cats and three control cats were then challenged orally and intranasally with a VP virus strain isolated in this laboratory. The vaccinated animals did not show symptoms, whereas the controls developed VP and died. The carcases were autopsied at the Department of Pathological Anatomy by Dr. F. VETÉSI.

## Clinical observations

*Symptoms.* Hyperacute, acute and chronic forms of VP could be distinguished on the basis of symptoms and course.

The hyperacute form is characterized by abrupt onset, marked listlessness, high fever (41—42 °C), fits of vomiting and exsiccosis, followed by subnormal temperature and death after 3—4 days. The leucocyte count fell to 1000—500/$\mu$l.

The acute form, the most frequent manifestation of VP, is characterized by marked listlessness, high temperature (40.5—42 °C), frequent, practically uninterrupted vomiting and a bile-like yellowish-green colour and slightly

foamy appearance of the vomitus. The cats show signs of great thirst; they often satisfy their extreme water requirement by supporting the chin on the rim of the water bowl and sipping water all day. Frequent vomiting results in a severe exsiccosis. The skin can easily be lifted anywhere over the body surface, but especially in the dorsal region. The creases remain well visible for several minutes (Fig. 4). The sick cats have a peculiar distracted gaze and scarcely respond to their environment. The retropharyngeal and submandibular lymph nodes are slightly swollen. The conjunctiva exhibits reddening and



*Fig. 4.* Severe exsiccosis caused by panleucopenia

there is a slight lacrimation. The breath is foul-smelling. Sometimes a regular arch-shaped necrotic zone, 3—4 mm wide, develops in the tongue mucosa, and finally becomes desquamated (panleucopenic marginal ulcer of the tongue) (Fig. 5). In part of the cases, above all in cats older than half to one year, a yellowish, purulent-appearing deposition, which can be wiped off, appears on the surface of the tonsils, which are red and swollen throughout the course of the disease. The oral changes are accompanied by a slight salivation. Swallowing is apparently painful. The cats refuse food for several days. Initially the intestinal tract seems empty on palpation; gas and fluid appear in it only after 4—5 days (a characteristic splash is heard on deep palpation of the stomach). At this time diarrhoea sets in and the markedly foul-smelling stool often contains blood streaks. Occasionally, the liver is slightly enlarged, accompanied by a scarcely noticeable yellowish coloration of the visible mucous membranes. One of the cats that yielded virus had liver dystrophy in addition to the other symptoms.

Among the laboratory examinations, haematological tests are very important. Parallel to the development of symptoms, the leucocyte count falls markedly. The leucopenia is occasionally very severe, with leucocyte counts

as low as 500—800/$\mu$l. The erythrocyte count does not notably change; it may eve rise above the physiological level, owing to the severe exsiccosis and haemoconcentration. Evaluation of the differential count is rendered difficult by the leucopenic state; thick smears are required to obtain proper information. The bulk of white blood cells are lymphocytes.

The urine contains trace amounts of protein and, if there is also liver dystrophy, an abnormal quantity of bile pigments, but no other pathological constituents.

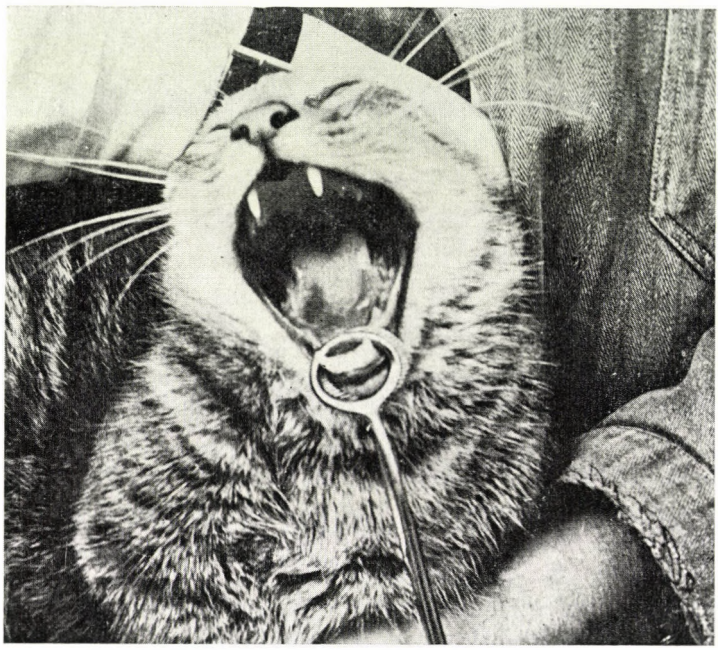

*Fig. 5.* Panleucopenic marginal ulcer of the tongue

Chronic VP is developed by cats which improve only temporarily, if at all, after the acute stage. Essentially it can be regarded as a protracted form of acute VP. The sick cats are in a poor general condition, lying mostly in stupor.

The inner temperature is usually normal, but a medium high, intermittent fever (39.6—39.9 °C) may occur. The skin is dry and flaccid, and a severe exsiccosis often develops. The visible mucous membranes, especially the conjunctiva and the soft palate, are pale. The tonsils are invariably enlarged and red. Appetite is poor, or irregular. The diseased cats consume some meat for 2—3 days, then refuse everything except water or tea for several days. There is a gradual loss of condition, sometimes resulting in a severe emaciation. At this stage vomiting ceases, but intestinal catarrh and diarrhoea

persist despite all therapeutic efforts. Proteinuria appears or, if already present, it rises to a concentration of 100—150 mg/100 ml. The erythrocyte count decreases markedly, to 2.5—3.5 million/$\mu$l, whereas the leucocyte count rises (!) above the physiological level. Differential counting shows an increased lymphocyte count.

*Diagnosis.* This can unequivocally be established on the basis of the characteristic clinical symptoms and haematological findings. Exclusion of a picornavirus infection may be difficult, but the main symptoms of these are respiratory (quick, difficult respiration, sneezing) rather than enteric, and although the leucocyte count may decline it does not exceed the degree common with viral diseases (1800—2000/$\mu$l). A survey of the main viral cat diseases is presented in Table II.

**Table II**

Survey of the main viral cat diseases

| Disease | Systematic position of causal agent | Morbidity rate | Mortality rate | Characteristic symptoms |
|---|---|---|---|---|
| Panleucopenia | Parvovirus | high | high | Marked listlessness, high fever, vomiting, diarrhoea, marginal lingual ulcer, severe exsiccosis |
| Rhinotracheitis | Herpesvirus | high | low or medium high | Sneezing, nasal discharge, conjunctivitis, occasionally ulcerative stomatitis (more often on hard than soft palate), rarely nervous symptoms |
| "Influenza" | Picornavirus (various serotypes) | high | low | Sneezing, nasal discharge, lacrimation, conjunctivitis, difficult respiration, microbronchitis, catarrhal pneumonia, enteritis |
| Rabies | Rhabdovirus | low | high | Marked excitation and aggressivity, trigeminal paralysis, difficult deglutition |
| Aujeszky's disease | Herpesvirus | low | high | Salivation, hiccup, marked dilatation of one or both pupils, agitated flagellar movements of the tail under constant crying, change of place. In typical cases ruthless scratching of the facial region. |

Bacterial enteritis can be excluded on the basis of absence of characteristic haematological findings.

On differentiating VP from non-infectious diseases, consideration should be given to gastroenteritis due to alimentary factors or licked-off hair and to obliteration of the intestine by a foreign body; these very frequent conditions cause neither fever nor leucopenia. The chronic, anaemic form of VP should be differentiated from the toxicosis caused by cumarin-type rat poison.

*Therapy of VP.* We propose the following therapeutic measures: blood from repeatedly vaccinated cats (hospital blood donors) should be administered

by slow intravenous injection immediately after admission, in a dose corresponding to the body dimensions (5—15 ml). The blood is taken from the donors by cardiac puncture. If exsiccosis is severe, Ringer solution containing 5% glucose should be administered intravenously and subcutaneously, and 10—20 mg vitamin C, 0.012 mg strophantin or 0.01—0.02 g caffeine should be given. If exsiccosis is medium severe, subcutaneous administration of fluid is sufficient.

With very severe exsiccosis, intravenous injection of the fluid is imperative (slowly, in small amounts), for cutaneous absorption is delayed, due to the poor circulation. In such cases the subcutaneously injected fluid accumulates in the lower pat of the chest as an "aqueous swelling" and may transform into an abscess. To prevent this complication, a small amount of crystalline penicillin or another water-soluble antibiotic should be added to the subcutaneously administered electrolyte solution. Secondary bacterial gastroenteritis is prevented by treatment with 0.25 g neomycin or Neo-Te-Sol powder in enterosolvent capsule or in bolus for 3—4 days. Bacterial infections of other organs are prevented or controlled by aqueous or oily chloramphenicol injection. To improve liver function, 300 $\mu$g vitamin $B_{12}$ is administered intramuscularly for several days and a 10% glucose solution orally on several occasions. Blood transfusion can be repeated at intervals of 2—3 days with half amounts, and intravenous and subcutaneous administration of fluid can be continued on a daily schedule. Camomile tea should be given instead of drinking water. The animals must not be fed until vomiting ceases. Fresh, uncooked beef or horse meat and minced chicken liver are the diet of choice. After completion of the antibiotic cure, Polyvitaplex coated pills should be given for 4—5 days, for vitamin supply. This treatment should be continued for at least one week after the symptoms have subsided.

## Prognosis

All forms of VP are severe and the prognosis is usually poor. Morbidity and mortality are both high. Outbreaks may kill all cats in a street or district of a town. Out of 1770 cats treated for VP in the Small Animal Hospital over a period of 10 years, 1011 (57.1%) died. The mortality rate was 77.8% in the age group of 2—3 months and 61.1% in that of 4—6 months. The chances of survival seemed relatively better in the age groups of 7—12 months and, especially, 1.5—2 years, the losses being 50 and 46.8%, respectively. The percentage of recoveries did not notably change with progressing age: *e.g.* the death rate was 58.3% in the 3-year-old age group.

Literary data (Catcott, 1964; Kirk, 1971) and our own experience unequivocally show that survivors acquire a lifelong immunity to VP.

## Prevention

In several countries, vaccines containing inactivated VP virus have been used for prevention. The optimal age for the first vaccination is 3 months. A booster dose should preferably be given at 6 months. Later a yearly booster dose is sufficient.

A more firm immunity can be established by vaccines containing live virus of attenuated in tissue culture, which is known to be more immunogenic than killed virus. The schedule is the same as above. Vaccination of pregnant cats with live viral vaccine is contraindicated, for the virus causes congenital abnormality in the foetuses, chiefly cerebellar ataxia. Natural infection during pregnancy may have similar consequences (KIRK, 1971).

## Conclusions

According to our own experience, VP is a a very frequent, often fatal cat disease. Out of a total of 7944 cats treated in the Small Animal Hospital over a 10-year period (1962—1971) 1770 (22.3%) had VP. Outbreaks are most frequent in the early spring and autumn season and incidence tends to decrease during summer (Fig. 1). As to age group distribution, kittens as young as 2—3 months may already contract the infection (9.94% of our VP patients belonged to this age group). These kittens were the progeny of pet cats which had not had VP. Susceptibility is very high at 4—6 months (24.3% of our cat patients from this age group had VP). The eruption of permanent teeth takes place in the same period and mass losses among kittens at this age have therefore been erroneously attributed to the dentition, especially in the country, where VP is known by a popular name referring to tooth trouble.

Incidence is at peak at 7—12 months of age (38.3% of hospitalized cats). This can be explained by sexual maturation and the related wanderings, *viz.*, an abrupt increase of exposure to infection. VP is still relatively frequent at 1.5—2 years of age (16.2% of hospitalized cats), but its incidence is quite low above 3 years (6.10%). A rare occurrence was observed between 4 and 8 years of age (2.0—0.34%); in these age groups only pet cats maintained in isolation from others became infected.

Part of the sick cats showed an arch-like, 3—4 mm wide marginal erosion of the lingual mucous membrane, extending over the sides and tip of the tongue. According to KIRK (1971), this lesion also occurs in rhinotracheitis or in mixed rhinotracheitis-VP invections, but according to our own experience, rhinotracheitis-associated oral lesions localize on the soft and hard palate rather than on the tongue. CHRISTOPH (1963) termed the tongue lesion as agranulocytotic lingual ulcer, we prefer the name panleucopenic ulcer.

Virus isolation and infection experiments have unequivocally shown that infectious feline laryngoenteritis and VP represent one and the same cat disease.

Therapeutically, neutralization of the causal agent by specific antibodies and replacement of white blood cells by intravenous administration of homologous blood are of decisive importance. It is remarkable that blood transfusion is scarcely referred to in the literature; the related publications advocate antibiotic therapy and, sometimes, fluid infusion, but do not seem to attach any importance to the control of leucopenia by blood transfusion. Another main requirement of successful therapy is an appropriate rehydration, if possible by the intravenous route.

One main reason of the high mortality rate seems to be the circumstance that the overwhelming majority of the cats are brought to the elinic in an advanced stage of disease, 3—6 days after onset. The owners usually mistake the disease for an "indigestion" or the cat itself hides for several days because of feeling severely ill; in either case, the critical first days are lost for therapeutic approach.

The spread of the disease could only be prevented by regular preventive vaccination.

## Summary

Clinical and epizootological studies on 1770 cats diseased in feline viral panleucopenia (VP) are reported. Three different clinical forms of VP were differentiated, efficient therapeutic measures were evolved and the seasonal fluctuation and age-distribution of VP were established. Virus isolation and vaccination experiments were performed.

Out of 7944 cats treated in the Small Animal Clinic of the University of Veterinary Science over the 10-year period from 1962 to 1971, 1770 (22.3%) suffered from VP. Outbreaks occurred above all in the early spring and autumn seasons. Incidence was highest among the 7—12 months old cats (38.8% of total incidence).

The disease is manifested in hyperacute, acute or chronic form. The acute form is the most frequent; this is characterized by marked listlessness, high fever (40.4—42.0°C), fits of vomiting, severe exsiccosis and leucopenia (leucocyte counts may be as low as 500—1000/$\mu$l). Some diseased cats show an arch-like, 3—4 mm wide necrotic zone in the lingual mucosa (panleucopenic marginal ulcer of the tongue). Chronic VP develops from the acute form and is characterized by a persistent intestinal catarrh, anaemia and cachexy.

The main therapeutic measures are the slow intravenous (!) infusion of blood from older or immunized cats, rehydration by intravenous and/or subcutaenous route and oral (neomycin), and intramuscular (chloramphenicol) antibiotic treatment for the prevention of secondary bacterial infections.

Both the morbidity and mortality of VP are high.

The viral agent who isolated from various organs of 6 of 47 cats which had died with symptoms of VP. The VP. virus was isolated in secondary cat renal epithelial cell culture.

The experimental infections and virus isolations have unequivocally proved that the conditions known as infectious feline laryngoenteritis and VP represent one and the same disease.

A formol-treated experimental vaccine prepared from VP virus propagated in secondary cat epithelial cell culture proved to be equivalent with the preparation "Felidovac" (Behringwerke).

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The address of the authors: Dr. Zoltán HORVÁTH, Dr. László PAPP 1078 Budapest, Landler J. u. 2; Dr. Adorján BARTHA, 1143 Budapest, Hungária krt. 21—25, Hungary.

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# ON THE SPECIES OF *PARAMPHISTOMUM* OF CATTLE AND SHEEP IN HUNGARY

By

O. SEY

Department of Zoology (Head: M. WÉBER), Teacher's Training College, Pécs

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Various *Paramphistomum* species are frequent parasites of ruminants in Hungary. As they are pathogenic and their systematics are problematic, closer studies on *Paramphistomum* are not only of a helminthological, but also of a veterinary hygienic importance.

No detailed investigations of *Paramphistomum* were conducted in this country until recently; formerly all such infections were attributed to *Paramphistomum cervi*. KOTLÁN (1958) was the first to demonstrate that also *P. muicrobothrim* Fischoeder, 1901 and *P. microbothrioides* Price et McIntosh, 1944 occurred in Hungary.

*Paramphistomum* species collected in nearly all parts of the country during the past years are described in this paper and a critical evaluation of the known species is presented.

## Materials and Methods

Materials collected in the central abbatoirs of all counties of Hungary except Fejér, Heves, Nógrád and Szabolcs-Szatmár were used. The majority of the material originated from Baranya county.

Parasites recovered from the rumen were fixed in Carnoy's solution of 70% ethanol and were stored in 70% ethanol until processing. The specimens were either embedded in paraffin and cut into 8—10 $\mu$ sections in the median saggittal plane, or cut with a freezing microtome into 0.5—1.0 mm sections, depending on the species. "*In toto*" preparations were also made. The preparations were stained with haematoxylin and eosin, azane or carmin. A total of 370 preparations (250 *P. daubneyi*, 28 *P. ichikawai*, 52 *P. leydeni*) were available for microscopic examination. Certain organs (testes, pars musculosa) of the parasites were dissected out and their structure was studied in stained and unstained preparations.

## Description of the parasites

The first well-founded systematics of *Paramphistomum* was elaborated by FISCHOEDER (1903), who based species differentiation on certain morphological and organic properties which, however, did not prove to be fully reliable in later studies.

LOOSS (1912) was the first to show that the structure of the genital end parts as well as other anatomical and histological properties can be utilized in differentiating species of highly similar morphology. On the basis of these considerations, NÄSMARK (1937) elaborated for *Amphistomum* species a systematic classification based on the histological structure of the pharynx, atrium genitale and acetabulum. NÄSMARK's systematic principles are now generally employed, although a classification based exclusively on histological features seems to be somewhat arbitrary and mechanical. Nevertheless, it made possible e.g. the specific revision of *P. cervi* (DURIE, 1951; SWART, 1954) and the precise definition of its geographical distribution; formerly it had been regarded as ubiquitous. After NÄSMARK, investigators have pointed out the shortcomings of species determination on an exclusively morphological and histological basis and suggested that it should be replaced by a biological definition, in which the evolution and structure of the species, the range and type of intermediate hosts, *etc.*, should also be taken into consideration.

Species determinations carried out in this laboratory, were based on NÄSMARK's principles, but were completed by certain morphological and organological data and the range of intermediate hosts was specified wherever possible. The species descriptions to follow are, apart from some new data, not original, but it seemed worth-while to include them in the general survey of *Paramphistomum* incidence in Hungary.

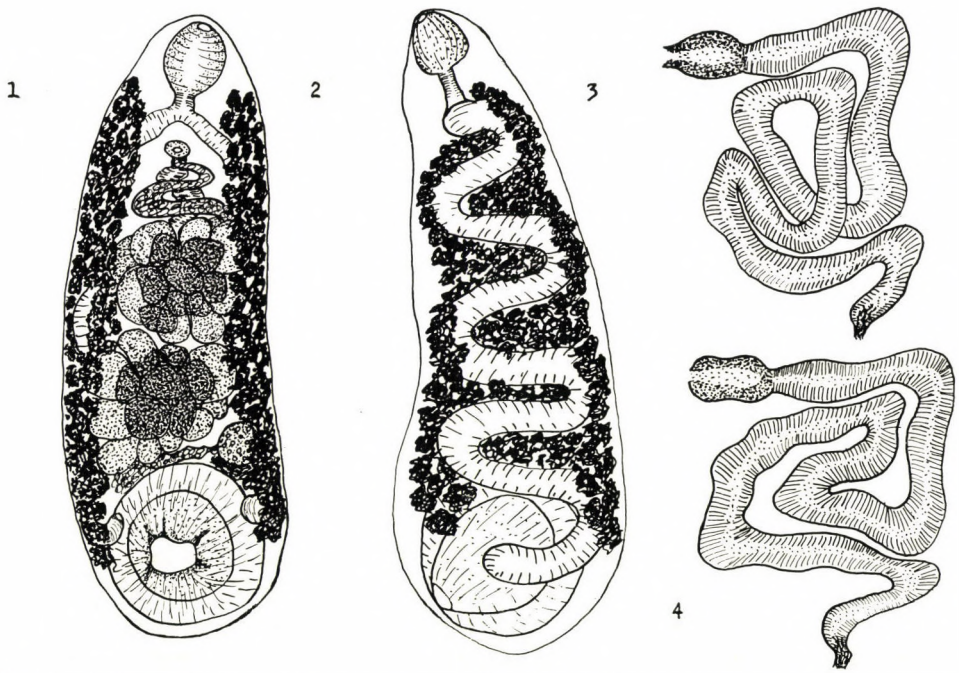
*Paramphistomum daubneyi* Dinnik, 1962 (Figs 1—24). The living parasite is of red colour, especially at the terminal parts. The smallest mature individuals are 4.6 mm, the largest ones 9.8 mm long, the average dimensions are 6.4 by 2.4 mm. The tegument has transversal folds, the conspicuous genital atrium is localized 1.1—1.9 mm away from the anterior end, roughly in level with the intestinal bifurcation.

According to DINNIK (1964; personal communication, 1972), the pharynx is of the *Calicophoron* type (Figs 6—10) and 1.0—0.5 mm long by 0.85—0.55 mm wide (average: 0.67 by 0.7 mm). Its position is terminal, but it may be deeply retracted; in the latter case a funnel-shaped empty space is seen between the pharynx and body end. The interior longitudinal muscular layer of the pharynx are 0.1—0.075 mm thick, the radial musculature 0.25—0.15 mm thick. The median circularly arranged muscle layer are conspicuous in some individuals and inconspicuous in others. The proportion of the pharynx to the full length of the body is 1 : 9. The oesophagus is always shorter than the

pharynx (0.7—0.3 mm long by 0.22—0.09 mm wide) and its shape and position may change, depending on the conditions of fixation. One or two thin muscular layers pass inside its wall and its surface is covered by a thick tegument which ceases abruptly at the level of the bifurcation of the oesophagus.

The branches of the caeca pass through the lateral part of the body, forming seven independent, dorso-ventrally oriented contortions. The terminal parts of the caeca always bend ventrally and end in level with the centre of the acetabulum (Fig. 2).

The testes are localized in the middle third of the body, one behind the other in a slightly diagonal position. They are 1.1—1.9 mm long by 1.6—1.7 mm wide, with a dorso-ventral length of 1.1—2.0 mm. They are deeply lobulated structures; the lobules range from 18 to 36 in number. Vasa efferentia passing from the testes join to form the vas deferens, which widens to form the thin-walled vesicula seminalis, consisting of numerous loops. The pars musculosa is well-developed and forms 3—7 contortions (Figs 3 and 4). The pars prostatica is tub-shaped, 120—300 $\mu$ long, and its greatest width corresponds to its length. The surrounding glands are moderately developed. The male genital organ communicates with the genital atrium through the ductus ejaculatorius. The genital atrium is of the *Microbothrium*-type. Diameter of genital opening is 0.5—0.75 mm. The genital fold, not always measurable, is 0.1—0.2 mm in




*Figs 1—4. Paramphistomum daubneyi. 1,* ventrally; *2,* laterally; *3—4,* the muscular and prostatic part of vas deferens

diameter, the genital chamber is 0.1—0.17 mm. The genital papilla can easily be recognized by its well-developed muscles. The genital sphincter is oval-shaped and 35—37 by 25—45 $\mu$ in size. The shape of the atrium genitale is changeable, depending on the physiological state in which the fixation was made (Figs 11—21).

The ovary is round, occasionally oval and 0.65—0.71 by 0.40—0.63 mm in size. It localizes in the space between the second testis and the acetabulum. The Mehlis gland is close by. The Laurer's canal passes from the ovary toward the dorsal surface of the body, and its opening is 1.6—3.2 mm away from the posterior end. The eggs measure 116—162 by 60—80 $\mu$.

The yolk glands form clusters on both sides of the body, extending from the pharynx to the centre of the acetabulum.

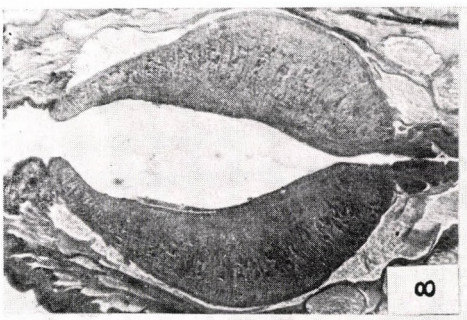
The excretory vesicle localizes between acetabulum, ovary, posterior testis and dorsal surface of the body; its aperture is 1.8—3.1 mm away from the posterior end.

The *Paramphistomum*-type acetabulum is in a subterminal position. Its outer diameter is 1.6—2.4 mm (average: 1.8 mm) in the dorso-ventral direction, its thickness is 0.25—0.65 mm and its aperture is 0.3—0.82 mm wide. The muscle units of the exterior dorsal series are often difficult to be identified, because the borderline between $de_1$ and $de_2$ is occasionally undefinable (Figs 22 and 24). The muscle units of $de_1$ range from 18 to 22, of $de_2$ from 20 to 30, of *di* from 37 to 54, of *vi* from 42 to 65 and of *ve* from 15 to 21. The average proportion of the acetabulum to the body length is 1 : 4.

Intermediate hosts: *Lymnaea truncatula* (Africa; Kenya, Ethiopia); *Lymnaea peregra* (Hungary). Snails reared in the laboratory after the conclusion of this report harboured fully developed cercariae.

*Discussion.* This is the first observation of the occurrence of *P. daubneyi* in Europe in general and in Hungary in particular. The comparison of our findings to descriptions by others and to the specimens supplied by Professor J. A. DINNIK unequivocally showed that the basic characteristics (position of gut branches, size of the atrium genitale, shape and passage of the pars musculosa and pars prostatica) of the species found in Hungary correspond in every respect to those of the parasite described in Kenya by DINNIK as *P. daubneyi*. The muscle units of the acetabulum are, however, dissimilar. This discrepancy can be ascribed to the loss of definition of the border between $de_1$ and $de_2$, because the appearance of the entire *de* series accord well with DINNIK's description. According to our observations, the measurements of the genital fold cannot be regarded as a reliable differentiating feature, because they may greatly be altered by the conditions of fixation (Figs 12, 13, 16 and 18). The extreme

*Figs 5—12. Paramphistomum daubneyi. 5,* fixed specimens; *6—10,* pharynx; *11—12,* genital atrium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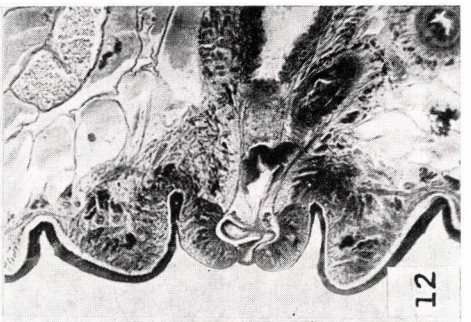
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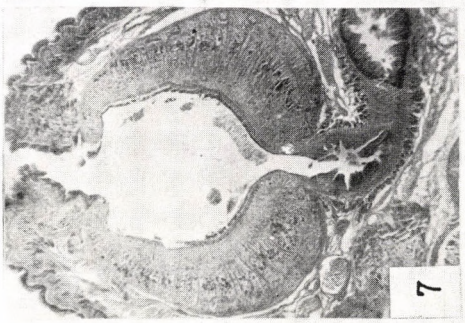
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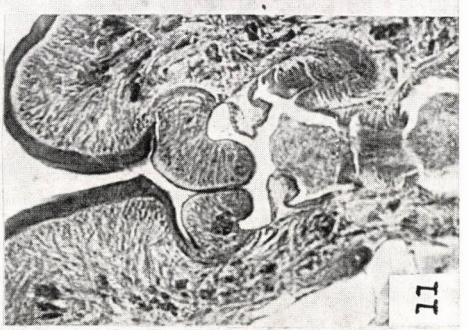
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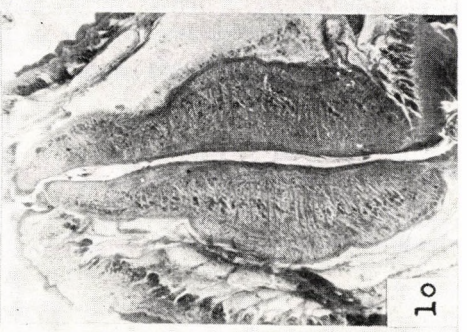
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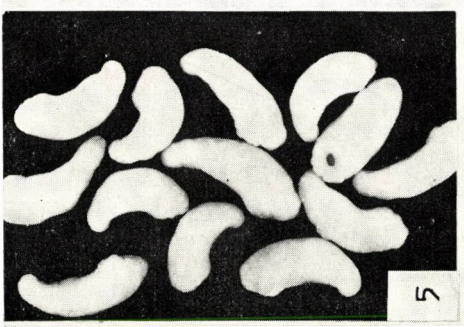
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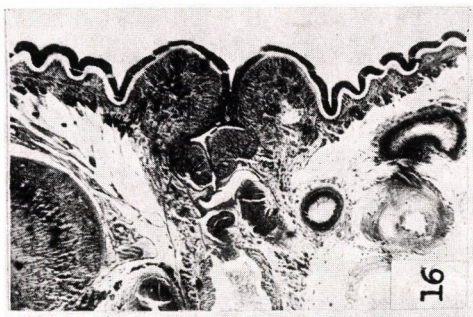
values of the dimensions of the pars prostatica and pars musculosa also vary in a wider range than specified by DINNIK. The measurements of the genital opening seem to be a safer differentiating feature and we, too, observed that the size of the genital sphincter is constant.

The occurrence of a *Paramphistomum* species with a *Microbothrium*-type genital atrium *(Paramphistomum microbothrium)* in Europe was first reported by NÄSMARK (1937) from Rome and by WILLMOT (1950) from a parasite material collected in France. The habitat of the Rome material was not described, thus its origin is doubtful. Similar species were later found in Europe by several authors (KOTLÁN, 1958; CAEIRO, 1961; MEREMINSKIY and VISHNYAKOV, 1969; ERHARDOVÁ, 1964; VUJIĆ, 1965; REINHARDT, 1969; SEY, 1971) who, except for REINHARDT, determined them chiefly on the basis of NÄSMARK's principles, without taking into consideration the differentiating features of closely related species. It is clear from DINNIK's reports (1961, 1962, 1964, 1965) that further to the features considered by NÄSMARK, other morphological characteristics and the knowledge of intermediate hosts are required for a precise species determination. We have taken into consideration, besides DINNIK's instructions the fact of snail host specificity as well.

It is known that the range of intermediate hosts of flukes is narrower than the range of their final hosts. The analysis of the intermediate host range of the known *Paramphistomidae* suggests that specificity for such hosts varies with the parasite species. E.g. *P. sukari* Dinnik, 1954 (DINNIK and DINNIK, 1957) develops exclusively in the snail *Biomphalaria pfeifferi (Planorbidae)*. The intermediate hosts of *P. ichikawai*, occurring in Japan, Australia, in the USSR and Europe, are *Segmentina alphena* (DURIE, 1953) in Australia and *Helicorbis sujfunensis* as well as *Gyraulus filiaris* (KISELEV, 1967) in the USSR, all *Planorbidae* snails. The European species *P. (= Liorchis) scotiae* is able to establish itself in ten different species of *Planorbidae* snails as intermediate hosts (NIKITIN, 1968; GLUSMAN, 1969). The specificity for groups (families, genera) of intermediate hosts seems to be a constant feature, because no *Paramphistomum* has hitherto been found to develop in intermediate hosts from two different families. *E.g.* DINNIK (1962) failed to infect families other than *Lymnaeidae (Bulinidae, Physidae, Planorbidae)* with the miracidia of *P. daubneyi* (intermediate host: *Lymnaea truncatula)* and *vice versa*, he could not infect *Lymnaea truncatula* with the miracidia of *P. microbothrium*, whose intermediate host is *Bulinus tropicus* southward from the Sahara.

Thus it seems fairly certain that the geographical habitat of *P. microbothrium* corresponds to that of its intermediate hosts, the snails of the family *Bulinidae*. As *Bulinidae* are not indigenous in Hungary, the species formerly described by KOTLÁN (1958) and SEY (1971) as *P. microbothrium* were in all

*Figs 13—20. Paramphistomum daubneyi.* Genital atrium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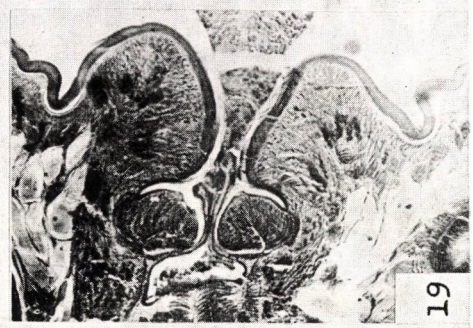
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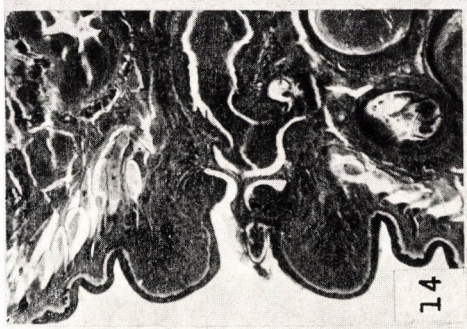
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probability misjudged *P. daubneyi*. Infection experiments performed in this laboratory with snails collected from a pasture infested with *P. daubneyi* (Mozsgó, Baranya county) were only successful with *Lymnea peregra* snails; *Planorbidae (Planorbis planorbis, P. spirorbis, Gyraulus crista)* did not become infected.

In Europe, *Paramphistomum* cercariae were first detected in Bulgaria (Popov *et al.*, 1967) in different snails of the family *Lymnaeidae (L. truncatula, L. palustris, L. peregra)*. Mereminskiy and Vishnyakov (1969) reported the occurrence of *P. microbothrium* in the same country; since, however, they recovered the cercariae from *Lymneidae* snails, the species seems to have been *P. daubneyi*.

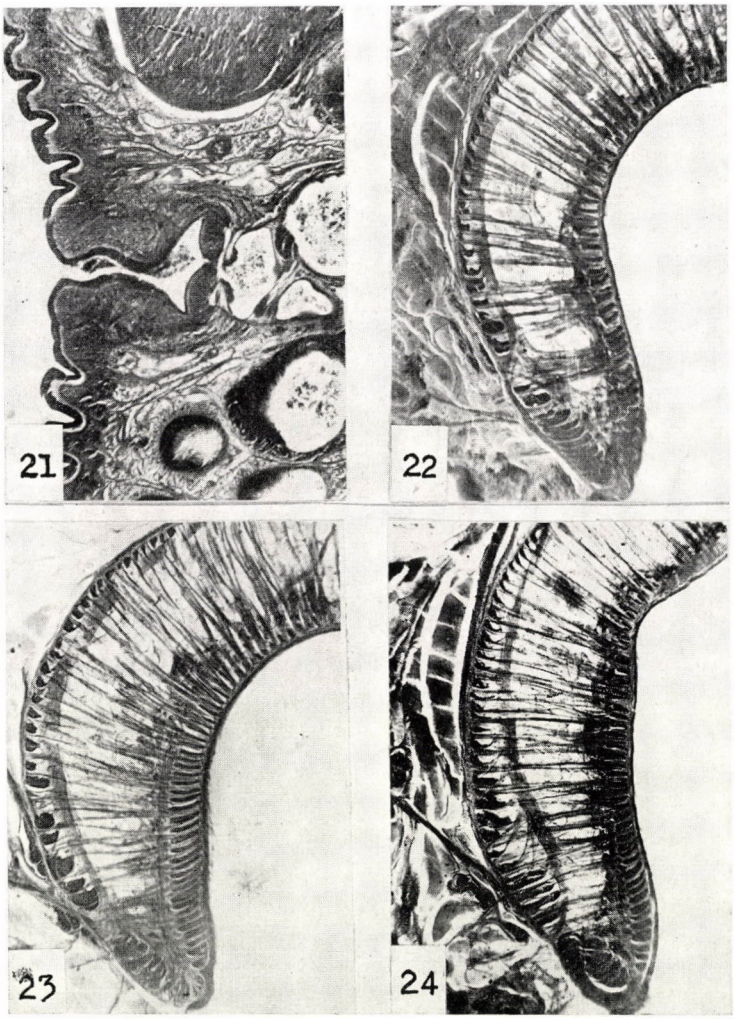
Apart from their specificity for different intermediate hosts, *P. microbothrium* and *P. daubneyi* can be differentiated from each other by certain morphological features, such as the orientation of the terminal portion of the gut branches, the dimensions of the atrium genitale, and the proportions of the pars prostatica and pars musculosa. The aforementioned Hungarian authors did not examine these characteristics when describing the Hungarian incidence of *P. microbothrium*.

The other species described in Hungary by Kotlán was *P. microbothrioides* Price and McIntosh, 1944. This species had been studied earlier by Bennett (1936), who referred to it as *Cotylophoron cotylophorum*.The short original description of Price and McIntosh (1944) discloses little information except that the $de_2$ is composed of 6—9 irregularly ordered muscle units, which are much fewer in number than the similar structures of the acetabulum of *P. microbothrium*. No other features required for species determination by Näsmark were considered; it can only be inferred that the pharynx and acetabulum were of the *Paramphistomum* type. Kotlán described a *Dicranocoelium*-type pharynx, but his illustration shows this to be a misinterpretation, as pointed out earlier by Velichko (1965). Price (1953) referred to the species again in a later report, stating that the main differentiating features are the number and arrangement of the fibres of the acetabulum. Lengy (1960) concluded on examination of specimens from Bennett's original collection that *P. microbothrioides* should be regarded as a synonym for *P. microbothrium*.

In view of the strict intermediate host specificity of *Paramphistomum* species, Lengy's suggestion can hardly be accepted. In the United States, the intermediate hosts of *P. microbothrioides* are *Fossaria parva* and *F. modicella* (Bennett, 1936), of the family *Lymnaeidae;* Hubendick (1953) classified them with *Lymnaea humilis*. On the other hand, the geographical distribution of *P. microbothrium* corresponds to that of the *Bulinidae* snails and there is no evidence of its being capable of developing in *Lymnaeidae*. These considerations strongly suggest that *P. microbothrioides* is an independent species, but do not exclude its identity with another *Paramphistomum* known under a differ-

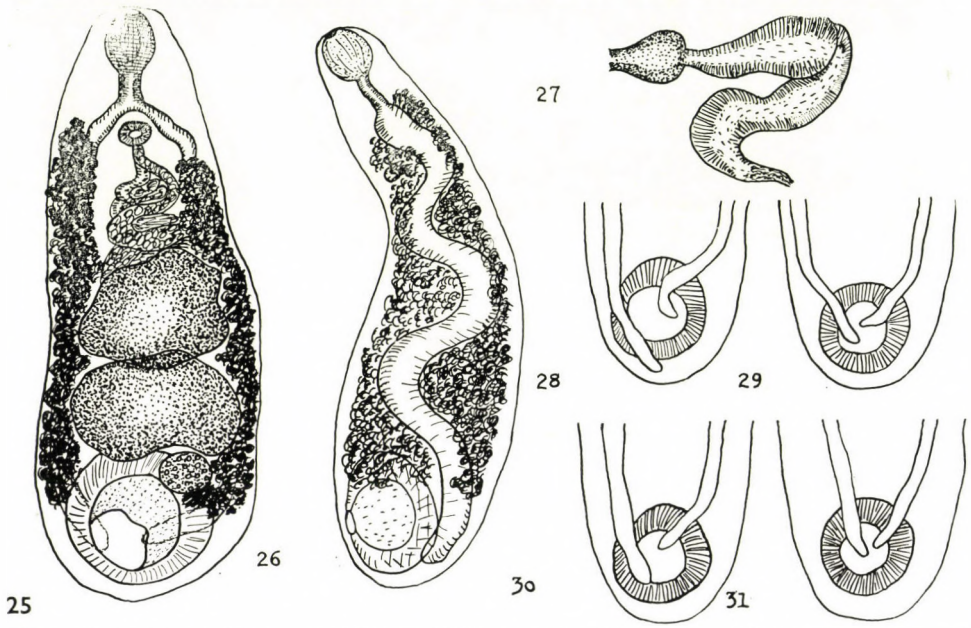
ent name. The clarification of this problem would require a thorough revision of BENNETT's work, with special regard to those organological properties which are indispensable for the differentiation of closely related species. These charracteristics were disregarded not only in the original descriptions, but also by those authors who studied the species in a greater detail (KOTLÁN, ERHARDOVÁ, LENGY).

*P. ichikawai* Fukui, 1922 (Figs 25—43). Specimens originating from cattle and sheep differ markedly in size (Figs 32 and 33). The largest parasites are 8 mm, the smallest ones 3.8 mm long (average 4.4 mm) by 1.5—2.3 mm wide. The living parasites are pale pink, also at the ends. The genital atrium is con-




*Figs 21—24. Paramphistomum daubneyi. 21,* genital atrium; *22—24,* acetabulum

spicuous, almost of the size of a sucker and it is 0.8—1.6 mm away from the anterior end. The *Paramphistomum*-type acetabulum is in a subterminal position; it is 1.0—1.5 mm thick, its outer diameter is 1.0—1.5 mm and the aperture is 0.2—0.7 mm wide. The dorsal exterior muscle units are sharply demarcated from one another and the muscle units of $de_1$ vary from 11 to 25, of $de_2$ from 6 to 18, of *di* from 34 to 54, of *vi* from 51 to 60 and of *ve* from 12 to 19. The proportion of the acetabulum to the body length is 1 : 3.




*Figs 25—31. Paramphistomum ichikawai. 25,* ventrally; *26,* laterally; *27,* the musculous and prostatic part of vas deferens; *28—31,* caudal end showing the position of the end of caeca and acetabulum

The *Calicophoron*-type pharynx (DINNIK, 1964) is in a terminal position. It is 0.4—0.7 mm long (average: 0.56) by 0.35—0.6 mm wide. The pharynx to body length proportion is on the average, 1 : 7. The oesophagus is shorter than the pharynx, 0.24—0.5 mm in length. The branches of the gut caeca pass in the lateral part of the body, forming five contortions in the dorsoventral direction (Fig. 26). The caeca are inequal, the left one being slightly longer than the right (Figs 28—31); they end dorsally and extend beyond the anterior margin of the acetabulum.

The testes lie adjacently in the middle third of the body; they are transverse oval-shaped, with usually intact margins, occasionally forming one or two lobules (Fig. 25). They are 0.8—1.5 mm long by 1.3—2.0 mm wide; the

*Figs 32—40. Paramphistomum ichikawai. 32—33,* fixed specimens; *34—35,* pharynx; *36—40,* genital atrium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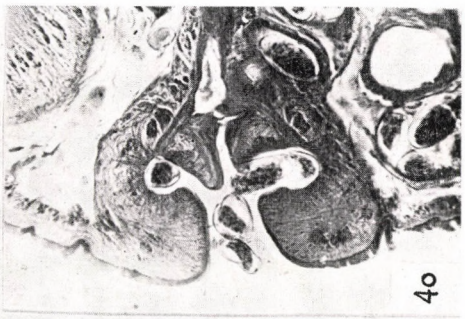
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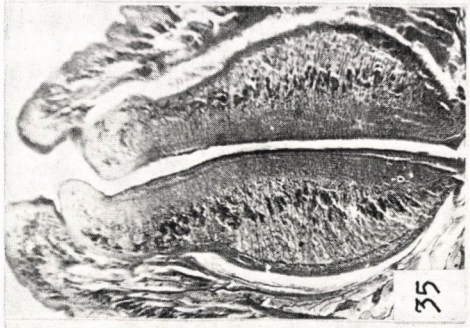
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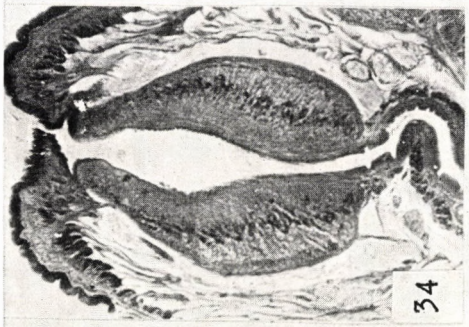
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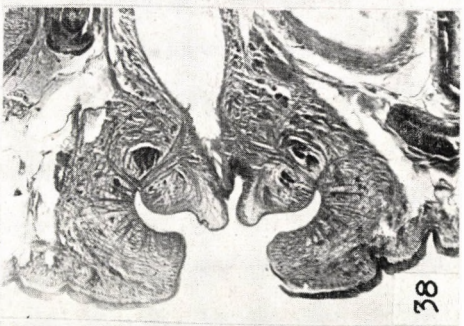
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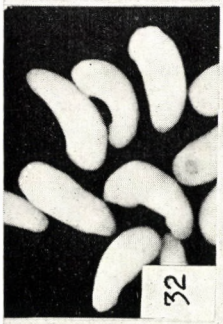
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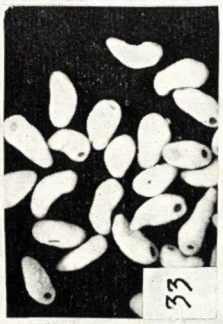
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dorsoventral length is 0.9—1.7 mm. The pars musculosa is moderately developed; it forms 2 or 3 contortions before it continues in the pars prostatica (Fig. 27), 0.15—0.35 mm long by 0.05—0.2 mm wide. The genital atrium is of the Ichikawai type (Figs 36—42); diameter of the genital opening is 0.4—1.1 mm; the genital chamber is 0.17—0.37 mm. The sphincter papillae is an irregularly shaped, non-compact, brush-like structure (Fig. 36).

The ovary is either round or slightly oval-shaped and localizes between the acetabulum and testis. It measures 0.6 by 0.4—0.5 mm. The Mehlis gland is closely associated with the ovary. The Laurer's canal passes from the ovary to the dorsoventral surface and its orifice is 1.3—1.7 mm away from the posterior end, always behind the excretory vesicle. The eggs are 135—140 by 69—75 $\mu$ in size.

The yolk glands localize in the lateral parts of the body and extend below the dorsal surface, but do not fuse.

The excretory vesicle occupies the space between ovary, acetabulum and body wall; its aperture is 1.8—2.5 mm away from the posterior end.

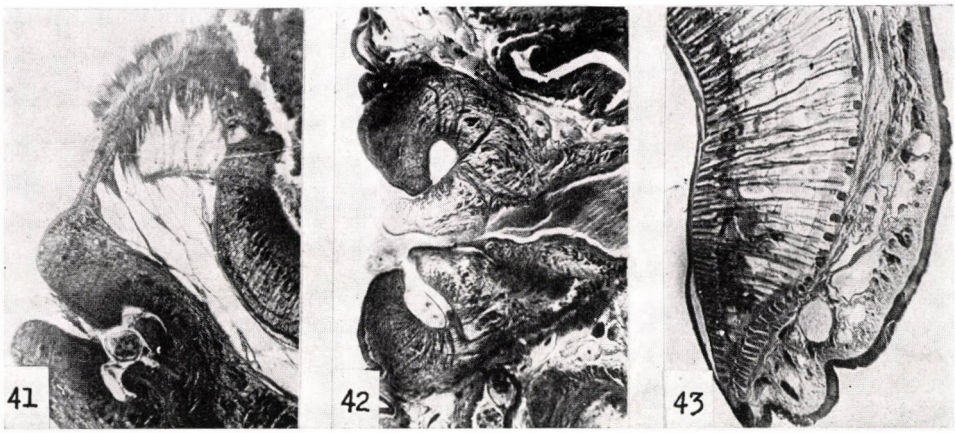
Intermediate hosts: *Helicorbis sujfunensis* (USSR) (unknown in Hungary)
*Segmentina alphena* (Australia)

*P. ichikawai* chiefly occurs in sheep; it was found in 7 of 11 infected sheep and in 4 of 97 cows.

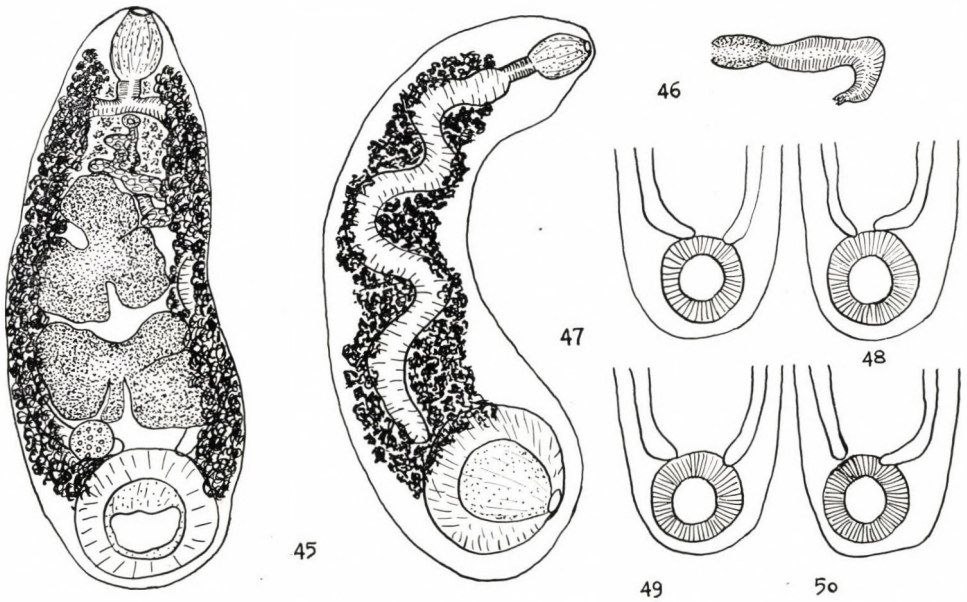
*Discussion. P. ichikawai* is very widely distributed and has several well-definable morphological features, such as the large, sucker-like genital opening shape of the testes, moderately developed pars musculosa, number and irregular arrangement of the $de_2$ muscle units of the acetabulum. Other features (body dimensions, number of other muscle units of the acetabulum do not notably differ from earlier descriptions (NÄSMARK, 1937; DURIE, 1951; KISILEV, 1967).

In Europe, it was first mentioned by VUJIC (1965) who, however, had not dealt with its morphology in greater detail.

*P. leydeni* NÄSMARK, 1937 (Figs 44—68). It is of medium size, and strongly bent ventrally. Specimens originating from cattle and sheep differ in size (Figs 51 and 52). The parasites are 4.0—7.0 mm (average: 5 mm) long by 1.3—2.0 mm wide; the dorsoventral length is 1.8—2.5 mm. The genital atrium is small, inconspicuous and localizes 0.6—0.9 mm away from the anterior end, at the plane where the gut branches off. The acetabulum is of the *Paramphistomum* type and opens ventrally. The $de_1$ and $de_2$ muscle units are sometimes separated (Fig. 67), sometimes linked by intermediate fibres (Figs 65 and 66). The same applies to the *ve* muscles. Muscle units in an intermediate position, 5—14 in number, were observed in 18 of the examined 38 parasites. The numbers of the acetabulum series are as follows: $de_1$ 9—16, $de_2$ 15—39, *di* 32—48, *ve* 12—24, *vi* 33—59. The acetabulum is 0.9 1.6 mm in diameter, 0.21—0.30 mm thick and its aperture is 0.2—0.7 mm wide. The acetabulum to body length proportion is on the average 1 : 3.5.




*Figs 41—43. Paramphistomum ichikawai. 41—42,* genital atrium; *43,* acetabulum




*Figs 44—50. Paramphistomum leydeni. 44,* ventrally; *45,* laterally; *46,* the muscular and prostatic part of vas deferens; *47—50,* the caudal end showing the position of the of caeca and acetabulum

The pharynx is of the *Liorchis* type (Figs 53—57) and its position may be terminal (Fig. 54) or retracted (Fig. 57), depending on the conditions of fixation. The papillae of the pharynx are moderately developed (25 $\mu$) and tapering posteriorly (Fig. 54). The interior longitudinal muscle layer is 150 $\mu$ wide, reaching to the middle circular muscle layer; anteriorly it extends up to four fifths of the pharynx (Fig. 54). The radial muscle layer is 50—55 $\mu$ wide. The

average pharynx for body length ratio is 1 : 8.1. The gut branches localize in the lateral part of the body, forming 4—5 contortions (Fig. 45). The terminal portions of the caeca pass dorsally and end at the anterior outer margin of the acetabulum (Figs 47—50).

The testes localize in the middle third of the body in a tandem position; they consist of 4—5 lobules, resembling sometimes a clover leaf (Fig. 44). The two testes are nearly identical in size, measuring 0.5—1.2 mm in length by 1.3—2.0 mm in width; the dorsoventral length is 0.2—2.0 mm. The pars musculosa and pars prostatica are both underdeveloped (Fig. 46); the former is 0.13—0.25 mm long by 0.055 mm wide, the latter forms a single curvature to join the vesicula seminalis.

The prostatic gland cells are well developed and form 2—3 rows. The genital atrium is of the *Epiclitum* type and both its shape and size are practically constant (Figs 58 and 62). The radial muscle fibres are well-developed. The relative position of the genital atrium end may be variable, depending on the conditions of fixation: the former may be 0.4—0.8 mm below the latter, in level with its centre (Fig. 62) or before it (Fig. 63). The full length of the genital opening is 0.4—0.5 mm, the genital chamber is 0.17—0.22 mm long.

The ovary localizes between the acetabulum, posterior testis and posterior branches of the gut. It is either round, or slightly oval and measures 0.5—0.7 by 0.45—0.6 mm. The Mehlis gland approximates the ovary in size. The Laurer's canal always opens dorsally, behind the aperture of the excretory vesicle, 1.5—2.0 mm away from the posterior end of the body. The eggs measure 140—156 by 75—80 $\mu$. The yolk glands localize laterally on both sides and extend over the dorsal surface in the anterior third of the body, until they fuse.

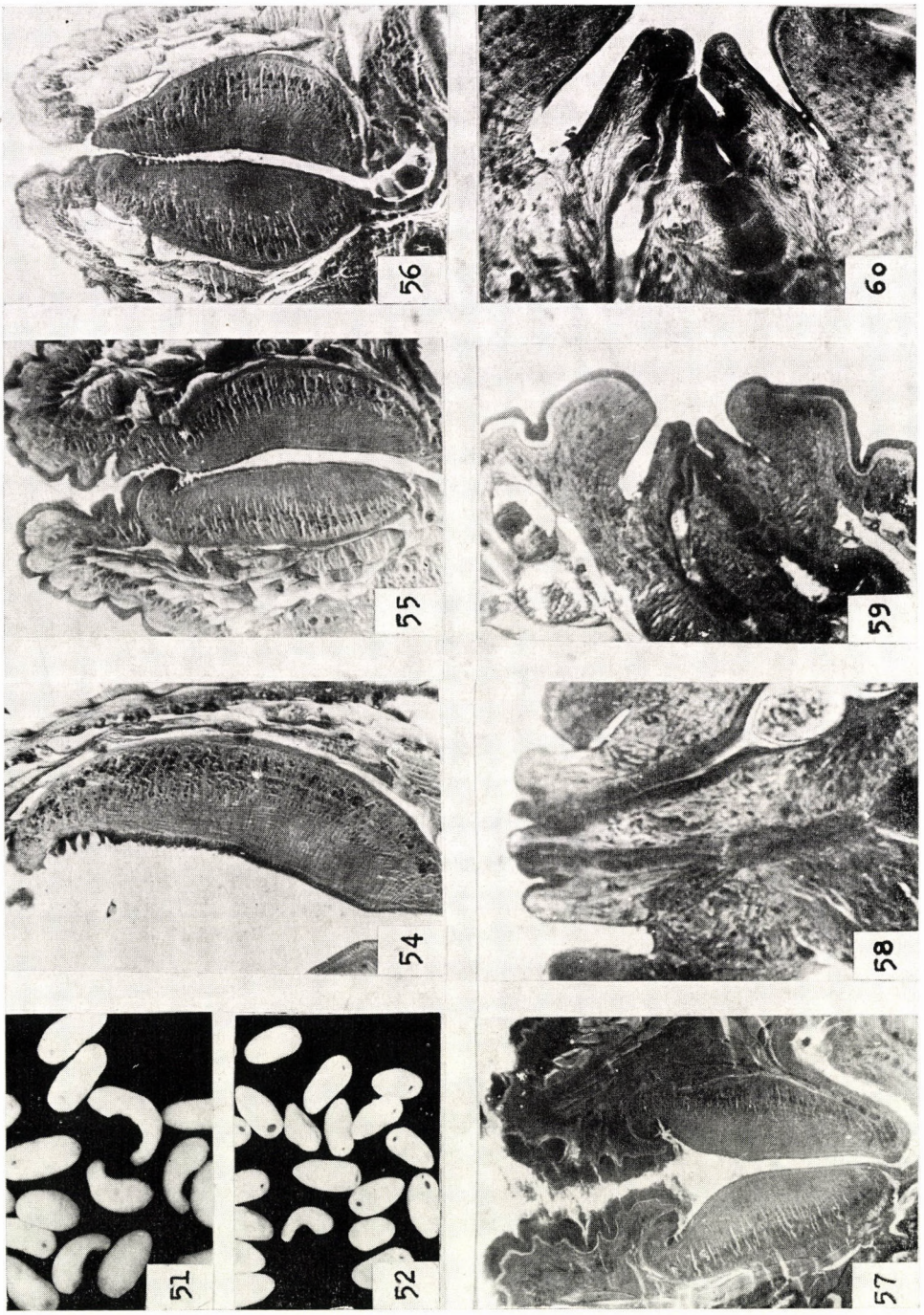
The excretory vesicle (Fig. 64) localizes dorsally between the ovary, acetabulum and body wall and its aperture is 1.7—3.6 mm away from the posterior end. Its distance from Laurer's duct is 0.9—1.1 mm

*P. leydeni* occurs both in cattle and sheep. It was recovered from 11 of 97 infected cows and from 6 of 13 infected sheep.

The intermediate host of *P. leydeni* is unknown.

*Discussion. P. leydeni* was originally described by NÄSMARK (1937) from a specimen kept in the museum of Leyden (The Netherlands) and from a test material originating from the Königsberg Zoo (Kaliningrad, USSR). NÄSMARK's description does not inform about the number of the muscle units of the acetabulum. VUJIĆ (1965) was the next to find the species in Europe and although he did not deal much with the morphological details, he gave the number of the $de_2$ units as about 39.

*Figs 51—60. Paramphistomum leydeni. 51—52,* fixed specimens; *54—57,* pharynx; *58—60,* genital atrium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WILLMOTT (1950) described two new species — *P. hiberniae*, *P. scotiae* — from test materials originating from Scotland, Ireland and the Netherlands. Both species have a modified *Liorchis*-type pharynx, but the atrium genitale of *P. hiberniae* is of the *Ichikawai*-type, that of *P. scotiae* of *Epiclitum* type.

VELICHKO (1966) found on examination of WILLMOTTs original material that *P. hiberniae* also has an *Epiclitum*-type atrium genitale and characterized its pharynx as pseudo-*Liorchis*-type. He created a new genus — *Liorchis* Velichko, 1966 — for WILLMOTT's two new species, but later (VELICHKO, 1971) she suggested that *P. hiberniae* should be regarded the synonym for *P. scotiae*.

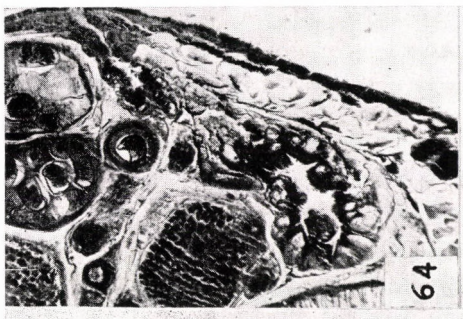
According to WILLMOTT (1950), the modified *Liorchis*-type pharynx differs from the common *Liorchis* type by the morphology of the middle and exterior circular muscles, these being very well developed in the posterior two thirds of the pharynx and inconspicuous in the anterior third.

The pseudo-*Liorchis*-type pharynx observed by VELICHKO (1966) in *P. hiberniae* is devoid of papillae and the radial muscle fibres are thicker and more refined as compared to the *Liorchis* pharynx. The *Liorchis*-type acetabulum differs from the *Paramphistomum*-type by division of the $de_1$ and *ve* muscle series into two parts; this means that the *de* series consist of three sharply delineated parts.

NÄSMARK characterized the middle and circular muscle series of the *Liorchis*-type pharynx as conspicuous in the posterior part and much less conspicuous in the anterior part. Our own experience is likewise that the middle circular muscle units are differently developed over the circumference of the pharynx being anteriorly inconspicuous and often associating with the exterior circular muscles in this part (Fig. 54). The papillae of the *Liorchis*-type pharynx tend to increase in the sequence *P. cervi*—*P. leydeni*—*P. gotoi;* those of *P. cervi* being quite small (NÄSMARK). VELICHKO does not inform in her report whether the *P. hiberniae* devoid of pharyngeal papillae originated from WILLMOTT's material or some other sample; in the latter case it might have been another species. As neither the *Liorchis*-type (WILLMOTT), nor the pseudo-*Liorchis*-type (VELICHKO) pharynx shows any constant difference from the structure described by NÄSMARK as LIORCHIS-type pharynx, they can well be regarded as individual variations of the latter.

As to the new type of acetabulum described by VELICHKO (1966), reference is made to NÄSMARK's observation that the inner circular $de_1$ and *ve* muscles of the *Paramphistomum*-type acetabulum sometimes form anatomical intermediate position to the outer parts of the same and to the circular $de_2$ muscles as well (p. 332), as for example in *P. leydeni*. We observed this phenomenon or, to put it according to VELICHKO, the threefold partition of the *de*

*Figs 61—68. Paramphistomum leydeni. 61—63,* genital atrium; *64,* excretory duct; *65—68,* acetabulum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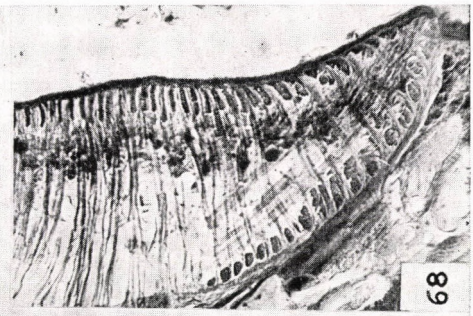
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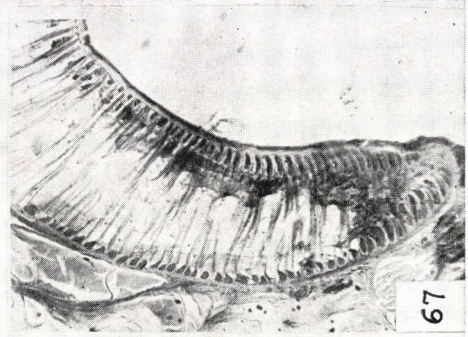
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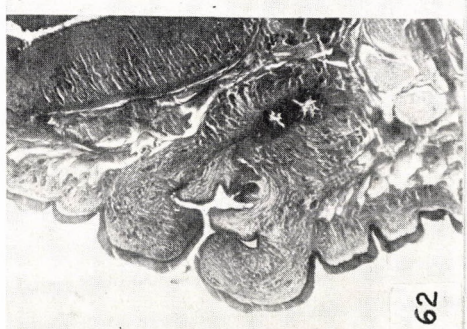
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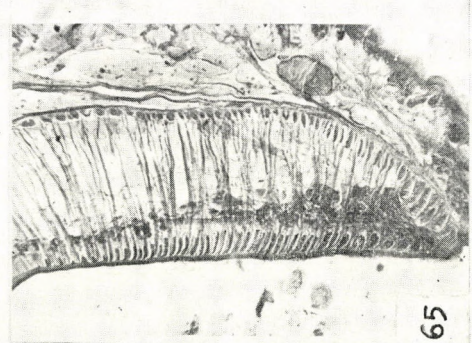
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muscles, several times in our own material (Figs 65 and 66), but did not regard it as a constant species characteristic. We therefore believe that VELICHKO's *Liorchis* acetabulum and *Liorchis* genus are entirely within the category outlined by NÄSMARK for the genus *Paramphistomum*.

According to WILLMOTT (1950), *P. scotiae* and *P. leydeni* can be regarded as synonymous as long as only the pharynx, genital atrium and acetabulum are considered, but certain other morphological features weigh in favour of their independence from one another. WILLMOTT compared nine such differentiating features in a table; among them the acetabulum to body length ratio, the position of the genital atrium and the length of the excretory duct differ notably, the rest seem to be less important and show great individual variation.

WILLMOTT calculated the acetabulum to body length ratio from the inner diameter of the former, thus the result was 1 : 6.4. If, however, the outer diameter is considered, as was visibly done by NÄSMARK, the ratio is 1 : 3.2, *viz.* almost identical with NÄSMARK's calculation for *P. leydeni* (1 : 3.6).

As already mentioned, the position of the atrium genitale depends greatly on the conditions of fixation, thus it is not of full value as a differentiating feature.

WILLMOTT (1950) refers to NÄSMARK in describing the excretory duct of *P. leydeni* as "very long", contrasted to the "very short" similar organ of *P. scotiae*. NÄSMARK, however, described the excretory vesicle as "very long" and made no reference to the length of the excretory duct (p. 461). WILLMOTT gave no numerical value either for the said length. On the basis of our own measurements we agree with NÄSMARK's opinion that the excretory vesicle can be regarded as long (0.75—1.2 mm), whereas the excretory duct is, like that of *P. scotiae*, short (150—200 $\mu$) (Fig. 64).

It follows from the above considerations that morphologically *Paramphistomum scotiae* is either very closely related to, or identical with, *Paramphistomum leydeni*. A definitive conclusion would, however, require closer morphological studies of certain organs of *P. scotiae* as well as the knowledge of the developmental cycle of *P. leydeni*.

## Summary

Three *Paramphistomum* species (*P. daubneyi* Dinnik, 1962, *P. ichikawai* Fukui, 1922, *P. leydeni* Näsmark, 1937), found in cattle and sheep in Hungary, are described. Out of them *P. daubneyi* occurred exclusively in cattle, *P. ichikawai* and *P. leydeni* chiefly in sheep and less often in cows.

It is demonstrated on the basis of specificity for intermediate hosts and morphological characteristics that the species described earlier in Hungary as *P. microbothrium* is in fact *P. daubneyi*.

The similarities between *P.(= Liorchis) scotiae* and *P. leydeni* are analyzed in great detail.

## Acknowledgements

The author is indebted to Dr. J. A. DINNIK for supplying the paramphistome species used for comparative study and to all colleagues who assisted him in collecting test material from different parts of the country.

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The address of the author: Dr. Ottó SEY, 7624 Pécs, Ifjúság u. 6, Hungary.

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# SEROLOGICAL STUDIES ON THE INCIDENCE OF VIRUSES ASSOCIATED WITH RESPIRATORY AND ENTERIC DISEASES IN CATTLE IN THE STATE FARMS OF FEJÉR COUNTY

By

E. Mocsári, Erzsébet Sághy, Julianna Kiss, E. Fülessy and L. Óváry

Central Veterinary Institute (Director: A. Ványi), Budapest, Veterinary Centre of Fejér County (Director: E. Lancz) and National Centre of State Farms, Department of Fejér County (Director: T. Brunner), Székesfehérvár

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The incidence of viral respiratory and enteric diseases in cattle has increased during the last years, due to the introduction of industrial-like technology (regrouping of animals, continuous re-stocking of calfwards, buying for fattening or stocking of newly established farms from different livestocks, etc.).

The incidence of different virus infections cannot be determined on clinical and epizootiological basis. Even the detection of the viral pathogen encounters difficulties in a considerable part of cases. At the same time an aetiological diagnosis would be necessary to introduce specific preventive measures against some viral diseases, especially because proper immunization and eradication programmes cannot be worked out without full knowledge of the incidence of viral infections. The same is valid for the preventive measures to be introduced in connection with buying and transporting animals.

In Hungary, serological screening tests were first carried out in the state farms of Fejér County. The presence of virus neutralizing antibodies to VD-, IBR- and adenoviruses as well as that of complement fixing antibodies to *Chlamydia* (syn. *Miyagawanella*, *Bedsonia*, Psittacosis-Lymphogranuloma-Trachoma (PLT) group) has been examined. The detection of neutralizing or haemagglutination-inhibiting antibodies to Paramyxovirus parainfluenza-3 seemed to be unnecessary because, according to previous results, antibodies to this virus were present in almost all large industrial farms (Csontos, 1970).

## Materials and Methods

*Blood samples* from 1998 cattle of 14 state farms in Fejér County were submitted for examination. Depending on the total number of animals 100 or 200 samples were examined from each farm; this amounted to 10—15% of

**Table I**

The incidence of antibodies against the viruses associated with respiratory

| Virus | Calves | | | Heifers | | |
|---|---|---|---|---|---|---|
| | examined | positive | | examined | positive | |
| | No. | No. | per cent | No. | No. | per cent |
| IBR | 548 | 15 | 2.7 ( 0—16)* | 395 | 14 | 3.5 (0—24) |
| VD | 523 | 183 | 34.9 ( 0—96) | 370 | 56 | 15.1 (0—44) |
| Adeno | 548 | 352 | 64.2 (12—96) | 395 | 255 | 64.6 (28—92) |
| *Chlamydia* | 548 | 55 | 10.0 (0—51) | 395 | 31 | 7.8 (0—36) |

* In parentheses, extreme percentages for individual farms

the total number of cattle. In the samples calves, breeding heifers, fatstock and cows were represented by the same number of sera, i.e., 25 or 50 from each farm. The sera were centrifuged, inactivated for 30 minutes at 56 °C and stored at —20 °C until tested.

*Virus.* In the serum-neutralization tests the Oregon $C_{24}V$ VD strain, the No. 610 IBR strain isolated in Hungary (CSONTOS and MACZKÓ, 1967) and Misk/65 adenovirus type 8 strain (BARTHA *et al.*, 1970) were applied.

*Tissue culture.* Examinations were carried out in primary (MONTES DE OCA *et al.*, 1971) and secondary (SCHWEIZER, 1966) calf testicle cell cultures prepared by trypsin-versene treatment. As growth medium Hanks' solution supplemented with 10% fetal bovine serum, as maintenance medium Hanks' solution without serum was applied.

*Virus neutralization test.* The viruses were diluted to a determinated 100 $TCID_{50}$/0.1 ml and were added to an equal volume of the sera. The sera were undiluted when tested against IBR virus and diluted 1 : 2 when tested against VD virus or adenovirus. Two tubes of primary or secondary calf testicle cell culture were inoculated with 0.2 ml of each virus-serum mixture previously incubated at 37 °C for an hour. The test was read 48 hours, 5 days and 7 days p.i. in the case of IBR virus, adenovirus and VD virus, respectively. As for the presence of virus neutralizing antibodies those sera were considered as positive which in the above-mentioned dilution entirely (VD and adenovirus) or — compared to the control — in 50% (IBR virus) inhibited the viral cytopathic effect.

*Determination of average antibody titre.* The test was performed in 10% of the positive sera with a quantity of 100 $TCID_{50}$/0.1 ml of virus and serial twofold dilutions of serum starting at 1 : 2. Equal volumes of these were incubated at 37 °C for an hour. A 0.2 ml volume of each mixture was inoculated into two primary calf testicle cell culture tubes. Final readings were made as described for the virus neutralization test.

and enteric diseases of cattle in the state farms of Fejér County

| Fatstock | | | Cows | | | Total | | |
|---|---|---|---|---|---|---|---|---|
| examined | positive | | examined | positive | | examined | positive | |
| No. | No. | per cent | No. | No. | per cent | No. | No. | per cent |
| 408 | 10 | 2.5 (0—28) | 647 | 256 | 39.5 (0—79) | 1998 | 295 | 14.8 (0—52) |
| 383 | 92 | 24.0 ( 0—84) | 622 | 299 | 48.1 (4—100) | 1898 | 630 | 33.2 (7—73) |
| 408 | 273 | 66.9 (36—100) | 647 | 434 | 67.1 (44—92) | 1998 | 1314 | 66.2 (34—86) |
| 408 | 43 | 10.5 (6—40) | 647 | 34 | 5.2 (0—24) | 1998 | 163 | 8.2 (0—32) |

*Complement fixation (CF) test.* The detection of antibodies against *Chlamydia* was carried out by CF test (CSONTOS and MÓCSY, 1962) using an antigen (BEER, 1965) produced by ether extraction and acetone precipitation from the yolk-sac of fertile eggs inoculated into yolk-sac. Complete fixation in a 1 : 5 serum dilution was considered as positive.

## Results

The results of examination of 1998 sera from 14 state farms of Fejér County are shown in Table I.

The average neutralizing antibody titres against IBR-, VD- and adenoviruses for the antibody-containing blood sera are shown in Table II.

**Table II**

The average neutralizing antibody titres against the IBR-, VD- and adenoviruses in positive sera samples

| Virus | Average antibody titre (log 2) |
|---|---|
| IBR | 1.37 |
| VD | 3.83 |
| Adeno | 4.12 |

In Table III the serological results for clinically healthy animals were compared with those for such livestocks in which viral respiratory and enteric diseases caused considerable losses during the period of examination.

**Table III**

The incidence of antibodies against the viruses associated with respiratory and enteric diseases of cattle in clinically healthy livestocks as well as in those in which the diseases have caused considerable losses

| Virus | Clinically healthy livestocks | Diseased livestocks |
|---|---|---|
| IBR | 13.57 (0—52)% | 16.16 (4—40)% |
| VD | 15.71 (7—31)% | 44.25 (7—73)% |
| Adeno | 55.71 (34—74)% | 73.41 (47—86)% |
| *Chlamydia* | 8.14 (0—28)% | 8.58 (0—32)% |

* In parentheses, extreme percentages for individual farms

## Discussion

On the basis of our serological examinations it can be concluded that among the viruses causing bovine respiratory and enteric diseases in the state farms of Fejér County the percentual occurrence of antibodies against adenoviruses and VD virus was much higher than that of antibodies against IBR virus or *Chlamydia*.

In the livestocks in which infectious respiratory and enteric diseases occurred, caused clinical symptoms and economic losses, antibodies against VD- and adenoviruses occurred in higher percentage than in those in which animals were healthy during and prior to the examinations. Antibodies against IBR virus and *Chlamydia* were found to occur in equal proportion both in clinically affected and symptom-free livestocks.

The results obtained show that from the viruses causing respiratory and enteric diseases in cattle adenoviruses and VD virus are the most wide-spread in the state farms of Fejér County and the same viruses play a primary role in the aetiology of these diseases. IBR virus and *Chlamydia* occur only in lower percentage and play a minor role in the infectious respiratory and enteric diseases of cattle.

## Summary

Serological tests were carried out in 14 state farms of Fejér County to detect virus neutralizing antibodies against VD-, IBR- and adenoviruses and complement-fixing antibodies against *Chlamydia*. From the 1998 blood samples examined antibodies were detected in the following percentage — varying from one farm to the other:

| | | |
|---|---|---|
| IBR virus | 0—52% | average 14.8% |
| VD virus | 7—73% | average 33.2% |
| adenovirus | 34—86% | average 66.2% |
| Chlamydia | 0—32% | average 8.2% |

The results of serological examination of blood samples from clinically healthy livestocks as well as from those clinically affected with viral respiratory and enteric diseases were compared. It is concluded that in the livestocks where the diseases occur and cause economic losses, antibodies against VD- and adenoviruses can be found in a higher percentage of the animals than in clinically healthy ones. No such difference was observed as for the occurrence of antibodies against IBR virus and *Chlamydia*.

It can be concluded that adenoviruses and VD virus play a more important role in the aetiology of viral respiratory and enteric diseases of cattle in the state farms of Fejér County than IBR virus and *Chlamydia*.

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Address of the authors: Dr. Endre MOCSÁRI, Dr. Erzsébet SÁGHY, Dr. Julianna KISS, 1149 Budapest, Tábornok u. 2; Dr. Emil FÜLESSY, Dr. László OVÁRY, 8000 Székesfehérvár, Csíkvári u. 17, Hungary.

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# A GOSLING DISEASE IN VIET-NAM

By

N. V. HANH*

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Using poultry as the main source of meat supply has various economic and other advantages in the developing countries. In Viet-Nam, the poultry and egg industry established with the aid of Hungarian experts is now evolving as an important branch of meat production. Intensively managed chicken, however, require large amounts of high-quality feed, some important constituents of which, above all the various vitamins and additives, have to be imported from abroad and their price adds considerably to the production costs.

Interest has therefore been centered on the development of goose breeding. Geese find the bulk of their food themselves by grazing in appropriate pastures and even under the conditions of large-scale management, they can be fed with the same fodder plants. In Viet-Nam, goose keeping is concentrated in the Northern Plane, above all in the surrondings of Hanoi, where the conditions are favourable. The greater part of geese belong to the indigenous breed, the minority are Chinese geese or crosses. There are two laying periods: the main season is the spring and an after-season period follows in the autumn.

## Materials and Methods

Organs of naturally diseased Vietnamese geese were shipped to Hungary for virus isolation. The experiments were performed under carefully controlled conditions to prevent contamination of the material by the Hungarian goose virus strain. The goose embryos and day-old goslings used in the experiments originated from a goose flock in which there had been no incidence of the viral gosling disease known in Hungary as "goose influenza" for the last three years.

Ten per cent organ homogenates were prepared from liver, spleen and kidney in a pH 7.4 PBS solution. An equal volume of chloroform was added to the organ suspension, the mixture was shaken for 15 minutes, allowed to

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stand overnight in the refrigerator and next morning it was centrifuged at 3000 r.p.m. The supernatant was placed in a Petri dish and air bubbles were blown into it by means of a pipette to remove residual chloroform. Then 0.2 ml amounts of the fluid were inoculated into the allantoic cavity of embryonated goose eggs preincubated for 10 days, by the technique proposed by DERZSY *et al.* (1970). The infected eggs were incubated at 37 °C and were candled 2 or 3 times daily. After the death of the embryo, the egg shell was disinfected with 0.5% nitrogenol; the egg was cooled at +4 °C, opened, the allanto-amniotic fluid was withdrawn and stored at —20 °C until used. Samples from each allanto-amniotic fluid were inoculated into serum-containing agar for bacteriological examination.

## Results

Vietnamese and Hungarian goose virus isolates were compared by cross neutralization tests in goose embryo. Antisera were prepared in growing geese; these were inoculated with the virus strain V twice at a 10-day interval and were exsanguinated 10 days after the second inoculation. The Hungarian strain B and hyperimmune sera to strains B, H, "Csontos" and LB were supplied by Dr. D. DERZSY, Budapest.

The neutralization tests were carried out in goose embryos. The virus material was diluted 1 : 5, 1 : 50, 1 : 500 and 1 : 5000 in PBS and an equal amount of antiserum was added to each dilution. After a thorough shaking, the mixture was allowed to stand at room temperature for 2 hours and 5 embryonated goose eggs were inoculated with each dilution. An appropriate control series was set up with each dilution, using broth instead of serum. Virus titres were determined by the method of Reed and Muench.

### *Epizootological observations*

The goose stock of Viet-Nam increased markedly during the last decade, but in recent years outbreaks of a previously unknown infectious disease have occurred in the flocks, like in the European countries. A sporadic incidence was first noted during the spring in 1966, again in 1967, and in 1968 the disease spread to many flocks, causing severe losses. Initially, losses occurred only among the 1—3 weeks old goslings, but recently also 4—5 weeks old birds have often become involved. In the infected flocks 60—90% of the birds died and in some farms, mortality even reached 100%.

Owing to the explosion-like spread of the disease and the extraordinarily high losses, the gosling disease was termed in Viet-Nam as "goose plague". The first minor outbreaks occurred in the Northern Plane, but later incidence was also noted in the mountain region and other distant areas of the country.

It seems that the agent was introduced everywhere by population movements and uncontrollable goose transports during the war. Mortality began to decrease with the spread of the disease to an ever growing area. It is believed that the chain of infection might have been broken by the mass losses and/or hard immunity might have developed. In 1970, there was only a sporadic incidence and no losses occurred.

The source from which the disease had been introduced into Viet-Nam could not yet be identified. A similar goose disease was observed earlier in China. Up-to-date large hatcheries do not yet exist in Viet-Nam; goslings are hatched under natural conditions and thus artificial hatching can be excluded as a factor of dissemination. Infected eggs, the carriers among layers and, probably, contamination with the virus of feed and instruments can be held responsible for the spread of the disease.

### *Clinical observations*

Under field conditions, goslings usually begin to show symptoms at 7—10 days of age, whereas on experimental infection often already after 3—4 days. Hyperacute cases, in which abrupt death was preceded only by a temporary listlessness, were observed in the initial stage of the outbreaks. The disease invariably set in with listlessness, inappetence and other signs of a febrile state. Water consumpton rose. Many birds showed symptoms indicative of a mild coryza, some had a difficult breathing, accompanied by a turbid nasal and oral discharge. Diarrhoea was rare. The stool was initially normal, later it became watery and white-greenisch. The birds huddled together in groups, lost condition until they were scarcely able to stand and died 2—3 days after onset. Both in natural and experimental infections, the disease took a protracted course in some individuals towards the end of the outbreak; in such cases exudation accumulated in the abdominal cavity, causing its dilatation.

In diseased flocks, losses usually reached peak at the end of the second week and at the beginning of the third week, after which mortality tended to decrease day by day and as a rule ceased during the fourth week.

### *Gross lesions*

In hyperacute cases, the post mortem findings were either negative, or only a few petechial haemorrhages were found in the serosa.

With a less rapid course, the main gross lesion was cardiac degeneration. In part of the cases dilatation of the heart, rounding of the cardiac apex, various degrees of myocardial degeneration and a greyish, cooked-meat-like colour of the heart were observed. Extravasation of a considerable amount of serum into subcutaneous connective tissue, thoracal, abdominal and/or

pericardial cavity was often found. The extravasated serum either remained liquid, or coagulated. Fibrin originating from the pleural and peritoneal exudation usually deposited on the surface of the liver, forming a thin or thicker coat over it (Fig. 1). In certain cases thick fibrin depositions of a loose texture were also found on the surfaces of abdominal and thoracal air sacs.

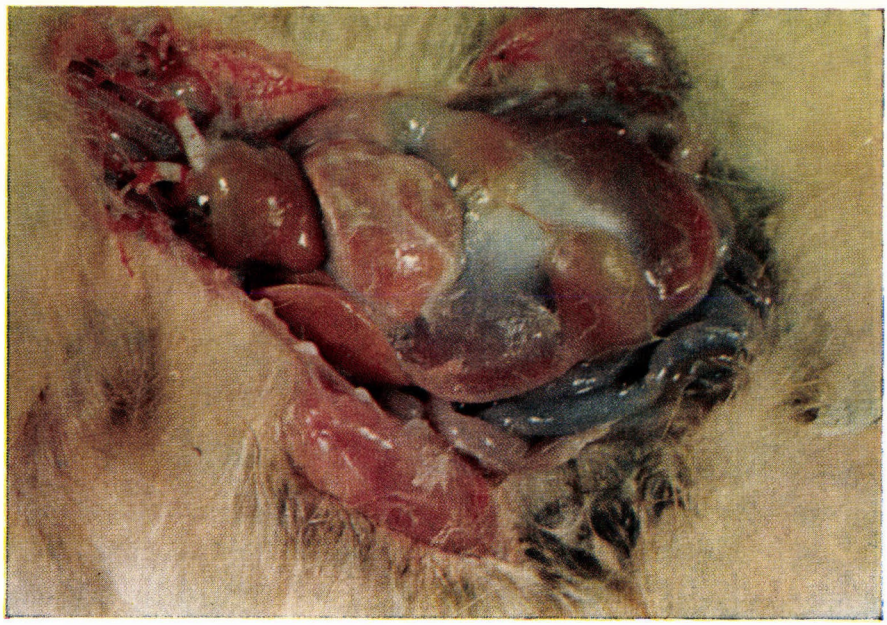

*Fig. 1.* Fibrin-coated liver of a 14-day-old gosling died in experimental infection

Lesions varying in type and severity were occasionally found in the digestive tract. Accumulation of mucin in mouth, oesophagus, proventriculus and, sometimes, in the gizzard, was a common finding. Intestinal changes ranged from a mild catarrh to severe inflammation. A fibrinous enteritis occurred with protracted courses of both spontaneous and experimental disease.

In some cases the liver was slightly hyperaemic and the gallbladder was swollen. Swelling of the spleen occurred only if there was a secondary bacterial infection (chiefly salmonellosis); in such cases yellowish-white necrotic foci appeared in liver and spleen. The laryngeal, pharyngeal and tracheal mucosa exhibited reddening. Mucosal hyperaemia and a viscous exudation were often found in the nasal and adnasal cavities. Pulmonary hyperaemia and oedematous infiltration of intralobular septa and pleura was a rare finding.

### *Microscopic lesions*

The most characteristic changes were found in the heart muscle. The muscle tissue was in places hyperaemic, oedematous and took on a pale stain. The myocardial cells lost transversal striation. The affected cells contained fat droplets and nuclear debris, and finally fell apart. The myocardial lesions were surrounded by inflammatory cells (Fig. 2). The pericardial connective tissue was initially oedematous, later infiltrating cells appeared in it.

The smooth muscle cells of the jejunum showed similar changes: degeneration and disintegration of the muscle tissue proper were followed by development of an inflammatory cellular reaction in the affected area.

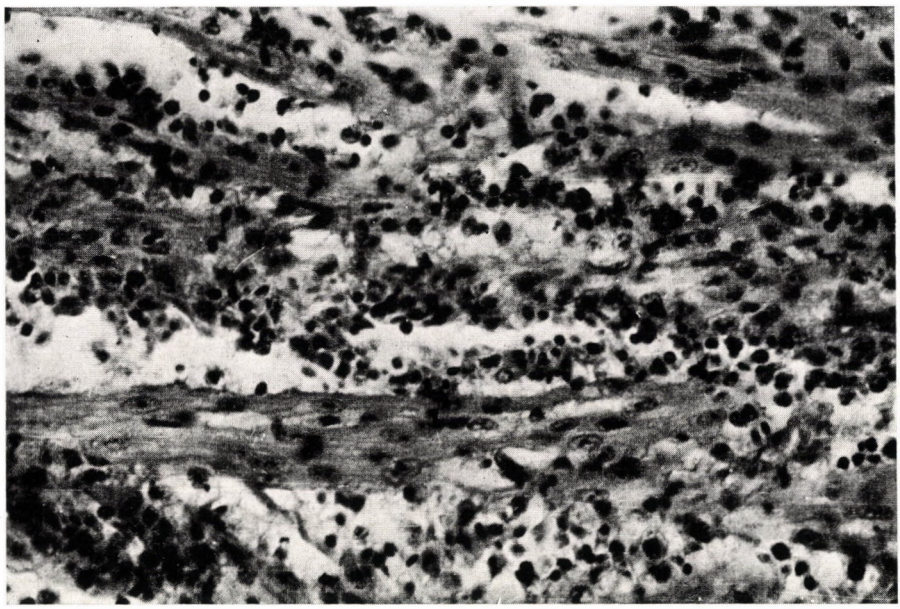

*Fig. 2.* Oedema and degeneration of myocardial cells. Note accumulation of mononuclear cells and erythrocytes between the muscle cells. H. & E., ×400

The mucous membrane of the jejunum deteriorated and became desquamated from the propria in many places. Infiltrating cells in the propria and hyperaemia of the submucosa were often seen.

The liver cells degenerated and stained pale with haematoxylin and eosin stain (Fig. 3).

### *Isolation and properties of the virus*

Several goose embryos inoculated with homogenates of gosling organs brought from Viet-Nam died between 3 and 8 days after infection. From the third passage, embryonic death became regular, occurring chiefly between

5 and 6 days. The main changes showed by the embryos were cutaneous hyperaemia, oedema and haemorrhages in the subcutaneous connective tissue, and myocardial degeneration. The virus titres of the allanto-amniotic fluids were low in the first passages, but reached a $5 \times 10^4$—$10^{4.5}$ $ELD_{50}$/ml value after the 5th passage. The isolate has been designated as "V".

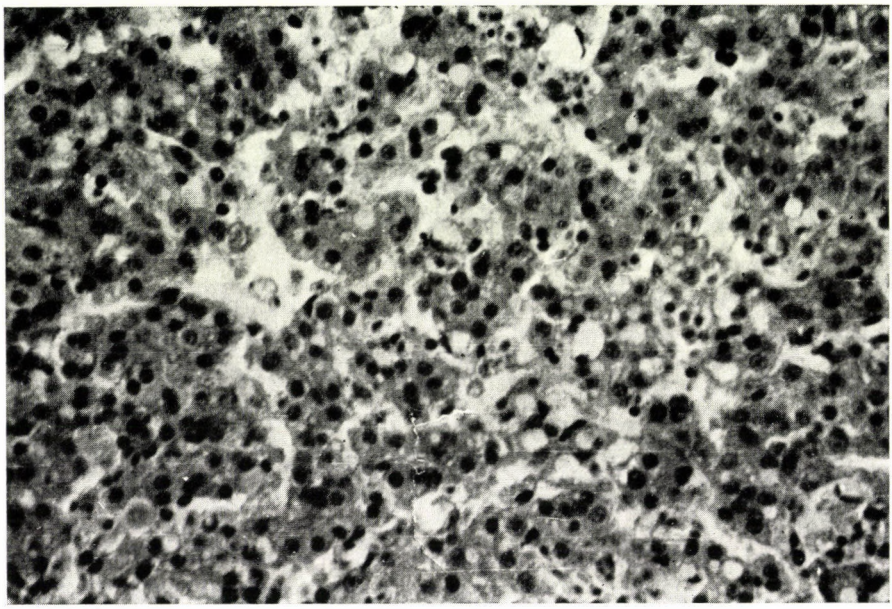

*Fig. 3.* Liver cell degeneration. The degenerated cells stain pale. H. & E., ×400

Virus strain V was equally well neutralized by homologous and anti-B serum, less well by anti-H and anti-Csontos sera and not at all by anti-LB serum. The Hungarian virus strain B was well neutralized by homologous and anti-V sera, slightly by anti-H serum and not at all by anti-Csontos and anti-LB sera (Table I). The antiserum prepared with the virus strain "Holland" (HOEKSTRA and SMIT, 1971) neutralized the isolates V and B at the same titre level as it did the homologous strain.

Serum protection and cross protection tests with the Hungarian and Vietnamese isolates were performed in goslings. Equal volumes of virus-containing allanto-amniotic fluid and antiserum were mixed, then thoroughly shaken and allowed to stand at room temperature. After incubation, 10 goslings were inoculated subcutaneously each with 1 ml of the virus–serum mixture. Ten control goslings, inoculated each with 0.5 ml virus containing fluid, were set up with each series.

In cross protection tests, goslings were uniformly protected by the anti-V and anti-B antisera (Table II).

**Table I**

Comparison of goose virus isolates from Viet-Nam (V) and Hungary (B) in neutralization test

| Virus strain | Serum | Virus dilution | | | | Virus titre 0.2 ml | Neutralization index |
|---|---|---|---|---|---|---|---|
| | | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | | |
| V | ∅ | 5/5 | 5/5 | 4/5 | 4/5 | $10^{4}$ | |
| V | anti-V | 1/5 | 1/5 | 0/5 | 1/5 | $10^{1}$ | 3 |
| V | anti-B | 1/5 | 0/5 | 1/5 | 0/5 | $10^{1}$ | 3 |
| V | anti-H | 4/5 | 4/5 | 2/5 | 1/5 | $10^{2.55}$ | 1.45 |
| V | anti-"Csontos" | 4/5 | 4/5 | 3/5 | 2/5 | $10^{3.34}$ | 0.66 |
| V | anti-LB | 4/5 | 4/5 | 3/5 | 5/5 | $10^{4}$ | 0 |
| B | ∅ | 5/5 | 4/5 | 4/5 | 3/5 | $10^{4}$ | |
| B | anti-B | 1/5 | 0/5 | 0/5 | 0/5 | $10^{1}$ | 3 |
| B | anti-V | 1/5 | 1/5 | 1/5 | 0/5 | $10^{1}$ | 3 |
| B | anti-H | 5/5 | 4/5 | 4/5 | 1/5 | $10^{3.35}$ | 0.65 |
| B | anti-"Csontos" | 5/5 | 5/5 | 5/5 | 5/5 | $10^{4}$ | 0 |
| B | anti-LB | 5/5 | 5/5 | 5/5 | 2/5 | $10^{4}$ | 0 |

The numerator designates the number of dead embryos, the denominator that of inoculated ones.

**Table II**

Cross protection tests in goslings

| Virus strain | Serum | No. | Goslings died % |
|---|---|---|---|
| V | | 4/10 | 40 |
| V | anti-V | 0/10 | 0 |
| V | anti-B | 0/10 | 0 |
| V | | 4/10 | 40 |
| B | anti-B | 0/10 | 0 |
| B | anti-V | 0/10 | 0 |

Propagation of strains V and B in goose embryo fibroblast culture was also attempted. Part of the cells became detached from the glass wall 6—7 days after infection, but this phenomenon seemed to be due to the aging of the culture rather than to a specific cytopathic effect (CPE). The third and fifth tissue culture passages of strains V and B were titrated in goose embryo. The results suggested that virus multiplication did take place in the tissue culture despite the lack of CPE: the titres were higher than originally (Table II).

Neither the chick embryo, nor the Peking duck embryo proved to be susceptible to virus strains V and B, whereas Muscovy duck embryos were

**Table III**

Behaviour of virus strains V and B in goose embryo fibroblast cell culture

| Virus strain and number of tissue culture passages | Titre observed | Titre calculated in absence of replication |
|---|---|---|
| $V_1$ | $10^3$ | $10^{3.5}$ |
| $B_1$ | $10^3$ | $10^{3.5}$ |
| $V_3$ | $10^3$ | $10^{1.5}$ |
| $B_3$ | $10^3$ | $10^{1.5}$ |
| $V_5$ | $10^{1.77}$ | |
| $B_5$ | $10^3$ | |

at least as susceptible as the goose embryos. Several times passaged V and B virus were equally lethal to the majority of Muscovy duck embryos, killing them within 2—5 days already in the first passage. After three passages, all Muscovy duck embryos died within 3—4 days and the virus could easily be reisolated from infected goose organs in Muscovy duck embryo.

The pathogenicity of virus strain V for day-old geese tended to decrease in the course of serial transfers in goose embryo. The mortality of goslings infected with virus V from the first passage was 47% decreasing to 40, 35 and 10% with materials from the 10th, 20th and 30th passage, respectively. Prolongation of the incubation period and reduction of the mortality rate both suggested a reduced viral virulence (Table IV) but, considering the low number

**Table IV**

Pathogenecity of virus strain V for day-old goslings after serial transfers in goose embryo

| Virus strain and passage number | No. of inoculated day-old goslings | Incubation in days Extreme (Mean) values | Losses | |
|---|---|---|---|---|
| | | | No. | % |
| $V_1$ | 36 | 4—15 (7.3) | 17 | 47 |
| $V_{10}$ | 20 | 6—17 (11) | 8 | 40 |
| $V_{20}$ | 17 | 16—17 (17.1) | 6 | 35 |
| $V_{30}$ | 20 | 10—17 (13.5) | 2 | 10 |

of the experimental birds and the great individual variation in the susceptibility of day-old goslings, the loss of virulence has remained to be confirmed by further experiments, the more as no similar decrease of virulence was observed with the Hungarian strain B.

The physical and chemical properties of strain V and B were found to

be identical. Both resisted chloroform treatment, pH 3, trypsin, saponin, sodium-dodecylsulphate, and heat treatment for one hour at 50 °C and 30 minutes at 56 °C (Table V).

**Table V**

Resistance of virus strains V and B to physical and chemical agents

| Treatment | Virus titre/0.2 ml after treatment | |
|---|---|---|
| | Strain V | Strain B |
| Control | $10^4$ | $10^4$ |
| Chloroform | $10^4$ | $10^4$ |
| pH 3 | $10^4$ | $10^4$ |
| Trypsin, 0.25% | $10^4$ | $10^4$ |
| Na-dodecylsulphate, 0.2% | $10^4$ | $10^4$ |
| Saponin, 0.1% | $10^{3.7}$ | $10^4$ |
| Mg** ion 50 °C, 1 hour | $10^4$ | $10^4$ |
| 50 °C, 1 hour | $10^4$ | $10^4$ |
| 56 °C, 30 min | $10^4$ | $10^4$ |

To obtain information on the size of the vision, samples of V and B virus were passed through Millipore filters of 220, 100 and 50 nm pore diameter. Since all filtrates and their 1 : 10 dilutions killed the majority of goose embryos, it has been concluded that the virion is smaller than 50 nm in diameter.

## Discussion

Incidence of an infectious gosling disease has recently been reported from several countries. In the German Federal Republic, the disease was described first as goose plague (KRAUSS, 1965), later as infectious goose hepatitis (SCHETTLER, 1971). The term "goose plague" had originally been coined in the Netherlands (VAN CLEEF and MILTENBURG, 1965; HOEKSTRA and SMIT, 1971). In the Soviet Union, KONTRIMAVICHUS *et al.*, (1966) described a disease causing mass losses in goslings as "infectious enteritis". In Hungary, the disease was named "goose influenza" (DERZSY *et al.*, 1970; CSONTOS and KIS CSATÁRI, 1967). Incidence of a similar disease was later reported from France (COUDERT *et al.*, 1972), Italy (RINALDI *et al.*, 1970) and Great Britain (CRIGHTON and WILKINSON, 1970).

Virological examinations resulted in the isolation of agents similar in physical and chemical properties to the Vietnamese strain V and the Hungarian strain B. At least part of the strains isolated in the different countries proved to be antigenically identical (V, B and "Holland").

The isolates can easily be propagated in goose embryo, but could not be adapted to chick and Peking duck embryos. The present studies have confirmed the observation of HOEKSTRA and SMIT (1971) that the Muscovy duck embryo is as susceptible as the goose embryo. The isolates multiply in goose embryo fibroblast cultures without causing a characteristic CPE, as observed earlier by KONTRIMAVICHUS and MAIBORODA (1968).

As to the systematic position of the goose virus isolates, several authors (DERZSY *et al.*, 1970; CSONTOS and KIS CSATÁRI, 1967; MANDELLI *et al.*, 1971) classified them as reoviruses, whereas SCHETTLER (1971) has regarded them as parvoviruses. There is experimental evidence that the virus passed the Millipore filter of 50 nm pore diameter, whence the virion seems to be smaller than 50 nm in diameter.

## Summary

The symptoms, gross and microscopic lesions associated with a disease recently causing great losses among goslings in Viet-Nam were found to be in every respect similar to those of the picture known in Hungary as "goose influenza". The disease, called "goose plague" in Viet-Nam, chiefly affects the one to three weeks old goslings and the mortality rate was initially as high as 60—90%. The symptoms and gross and microscopic lesions are described in great detail. In Viet-Nam, the spread of the gosling disaese was due to uncontrolled movements of persons and animals under the emergency conditions of war. Large hatcheries, similar to those representing the main sources of dissemination in Europe, do not yet exist there.

A virus strain designated "V" was isolated from organs of naturally diseased Vietnamese geese. The strain V and the Hungarian isolate B can both be propagated in goose embryo fibroblast culture without causing a well-defined cytopathic change. Both strains are resistant to chloroform, pH 3, heat, 0.25% trypsin, 0.2% sodium-dodecylsulphate and 0.1% saponin. Strain V tended to lose virulence after serial passages in goose embryo.

Virus neutralization and serum cross protection tests have indicated that the Vietnamese strain V, the most frequent Hungarian isolate B and a strain isolated from goslings in the Netherlands ("Holland") are antigenically identical.

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# POPULATION GENETIC STUDIES OF SERUM TRANSFERRIN VARIANTS IN SOME POPULATIONS OF HUNGARIAN SPOTTED CATTLE

By

P. Soos, Erzsébet Gippert and G. Csontos

Department of Immunogenetics, Centre for Artificial Insemination
(Director: I. Mészáros), Budapest

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**The discovery of foetal loss due to maternal-foetal differences has focused attention, beyond the classical case of Rh blood group system, on the role of other blood-group and polymorphic protein systems in natural selection.**

Hirszfeld and Zborovszki (1926) were the first to show deficiency of group-A children in families where the mother belonged to group 0 and the father to group A. Their finding has been confirmed by several authors with respect to group B, too. Levine (1943) and Wiener *et al.* (1949) found the frequency of spontaneous abortion to be related to the AB0 incompatibility between parents, but this findings has not been confirmed by others. Nevertheless, many contradictory findings were published in connection with fertility and AB0-Rh incompatibility.

In domestic animals maternal-foetal incompatibility on the basis of blood group incompatibility was first demonstrated in pigs by Szent-Iványi and Szabó (1954). Furthermore, Ashton (1958; 1959; 1961), as well as Ashton and Fallon (1962) described evidence for an effect of the transferrin locus on the fertility and embryonic mortality in cattle. Khattab *et al.* (1964), examining the transferrin system of sheep, concluded that the effect of the $Tf^C$ gene favours maternal-foetal incompatibility causing disturbed segregation rather than selective fertilization. This finding was confirmed also by Fésüs (1971) in the case of $Tf^I$; $Tf^A$ and $Tf^E$, and by Fésüs and Rasmusen (1971) in the case of $Tf^B$. In pigs Kristjansson (1964) showed that matings of $Tf^B$ homozygous boars to $Tf^A/Tf^B$ heterozygous sows resulted in a significantly higher return rate to service than other matings involving these two alleles. An excess of heterozygous genotypes was found by Imlah (1970) in all litters from matings of pigs being heterozygous for the $Tf^C$ allele. Furthermore, in pigs a consistent effect of alleles at the H blood group system and reproductive performance was found by Jensen *et al.* (1968). Morton *et al.* (1965) noticed that egg-white loci were associated with embryonic mortality in chickens.

Ashton and Dennis (1971) summarized the selective effects which may cause a difference in the production of progeny as follows: (1) zygotic selection, (2) embryonic selection, (3) prenatal selection, (4) postnatal selection, (5) adult selection, (6) prezygotic selection. They published data on litter-size, distribution of litters, sex ratio segregation collected from 13 generations of a laboratory mouse colony segregating for two murine transferrin alleles, $Trf^a$ and $Trf^b$. The overall analysis shows the $Trf^b$ allele with a 2.3% advantage over $Trf^a$ under defined conditions.

Numerous workers have gathered and published segregation data for transferrin alleles in various cattle breeds. Ashton (1965) evaluated a number of studies and concluded that matings from which equal number of offspring like and unlike the mother were to be expected resulted in a significant excess mother-like progeny. Earlier, Ashton and Fallon (1962) described the pooled data for 2544 inseminations between bulls and cows with different transferrin genotypes. The effectivity of matings between unlike homozygous, like homozygous, homozygous-heterozygous and heterozygous animals were 61.6; 55.6; 49.2 and 45.6%, respectively. These results indicated that heterozygotes were more viable *in utero*. Ashton

(1965) suggested that four phenomena affecting reproductive performance are associated with the transferrin polymorphism of cattle: (1) superiority of heterozygotes *in utero*, (2) maternal-foetal incompatibility, (3) differential fertility of homozygous and heterozygous bulls and (4) differential fertility of homozygous and heterozygous cows. Instead of maternal-foetal incompatibility COOPER and RENDEL (1968) used the more neutral term "unlike — homozygous — mother effect".

Many cases of small ratios of disturbed segregation of the cattle transferrin alleles have already been described (see: GAHNE, 1967; for references), but the insufficient number of observations and the large random error in each case did not allow to come to a positive or negative conclusion.

The purpose of this investigation was to examine the occurrence of the above-mentioned effects in our considerably large population analyzed for transferrin polymorphism. Furthermore, we wish to demonstrate these effects by taking into consideration also the distinction of the $Tf^{D_1}$ and $Tf^{D_2}$ alleles, as most of the papers have made no distinction between these two transferrin alleles and referred to them as $Tf^{D}$ only.

## Materials and Methods

Blood samples were obtained from 18,816 parentage-controlled individuals belonging to the Hungarian Spotted (Felckvieh) cattle breed.

For the determination of transferrin phenotypes zone electrophoresis in starch gels described by MAKARECHIAN and HOWELL (1966) was applied. Serum samples adsorbed onto Wathman 3M filter paper stripes were placed in an incision 3 cm from the cathode end of the gels. The inserts were removed after applying 150 V for 20 min. Electrophoresis was then continued using 300 V output voltage until the borate boundary had migrated 7.2 cm beyond the insertion line. The gels were stained with Amidoblack 10B — nigrosin solution and destained in methanol–water–acetic acid (5 : 5 : 1) mixture.

## Results

In the present material six transferrin alleles occurred and 14 phenotypes were observed. In addition to the four commonly occurring alleles ($Tf^{A}$; $Tf^{D_1}$; $Tf^{D_2}$ and $Tf^{E}$) two alleles, *viz.* $Tf^{F}$ (KOVÁCS, 1968) and $Tf^{I}$ Hungary (SOOS et al., in preparation) are present in Hungarian Spotted cattle. The allelic frequencies and the observed and expected distribution of phenotypes in the populations of A. I. bulls, cows and their progeny according to the HARDY-WEINBERG expectation are shown in Tables I and II. Table III demonstrates the distribution of transferrin phenotypes of progeny from such matings where equal numbers of progeny with transferrin types like and unlike the dams were expected. The present data show that the so-called "unlike-homozygous-mother effect" (COOPER and RENDEL, 1968) may cause a significant deviation ($P < 0.001$) from the expected 1 : 1 ratio of homozygous and hetero-

**Table I**

The gene frequency values of the transferrin alleles in the different populations.

| Population of | Number of animals examined | $Tf^A$ q | $Tf^{D_1}$ q | $Tf^{D_2}$ q | $Tf^F$ q | $Tf^E$ q | $Tf^I$Hungary q |
|---|---|---|---|---|---|---|---|
| A. I. bulls | 484 | 0.2200 | 0.1302 | 0.6043 | | 0.0455 | |
| Cows | 9 166 | 0.1948 | 0.1663 | 0.6155 | 0.0005 | 0.0228 | 0.0001 |
| Progeny | 9 166 | 0.1567 | 0.1819 | 0.6258 | 0.0002 | 0.0353 | 0.0001 |
| Total | 18 816 | 0.1769 | 0.1729 | 0.6203 | 0.003 | 0.0295 | 0.0001 |

**Table II**

Observed and expected distribution of transferrin phenotypes in different populations of Hungarian Spotted cattle according to the HARDY—WEINBERG's expectation

| Phenotypes | Population of | | |
|---|---|---|---|
| | A. I. bulls | Cows | Progeny |
| AA | 27 (23.431) | 340 ( 347.823) | 236 (225.070) |
| $D_1D_1$ | 9 (8.202) | 248 (253.484) | 300 (303.281) |
| $D_2D_2$ | 183 (177.701) | 3503 (3472.245) | 3585 (3589.641) |
| FF | | — (0.002) | — (0.001) |
| EE | — (1.003) | 5 (4.765) | 12 (11.422) |
| $I_HI_H$ | | — (0.001) | — (0.001) |
| $AD_1$ | 26 (27.732) | 627 (593.916) | 527 (522.530) |
| $AD_2$ | 119 (127.735) | 2159 (2197.996) | 1781 (1797.688) |
| AF | | 5 (1.785) | 1 (0.575) |
| AE | 14 (9.691) | 100 (81.420) | 92 (101.414) |
| $AI_H$ | | — (0.357) | — (0.287) |
| $D_1D_2$ | 76 (76.172) | 1865 (1894.752) | 2097 (2086.786) |
| $D_1F$ | | 2 (1.524) | 2 (0.667) |
| $D_1E$ | 6 (5.730) | 59 (49.343) | 108 (117.711) |
| $D_1I_H$ | | — (0.305) | — (0.333) |
| $D_2F$ | | 3 (5.642) | — (2.294) |
| $D_2E$ | 24 (26.613) | 248 (257.260) | 424 (404.967) |
| $D_2I_H$ | | 2 (1.128) | 1 (1.147) |
| FE | | — (0.209) | — (0.129) |
| $FI_H$ | | — (0.001) | — (0.001) |
| $EI_H$ | | — (0.042) | — (0.065) |
| Total | (484.000) | 9166 (9166.000) | 9166 (9166.000) |
| | $x^2 = 4.67$ (df.: 4) | $x^2 = 18.84^{**}$ (df.: 7) | $x^2 = 9.04$ (df.: 6) |

* df., degree of freedom = number of classes — number of classes pooled — number of gene frequencies — 1.

** $P < 0.05$ Expected values are in parentheses.

**Table III**

The "unlike-homozygous-mother effect", i.e. dams are homozygous for transferrin locus

| Matings ♂×♀ | N° | Homozygous progeny | Heterozygous progeny | $\chi^2$ | P (df.:1) |
|---|---|---|---|---|---|
| $AD_1 \times AA$ | 18 | 10 (9) | 8 (9) | | |
| $AD_2 \times$ | 66 | 33 (33) | 33 (33) | | |
| $AE \times$ | 4 | 2 (2) | 2 (2) | | |
| $AD_1 \times D_1D_1$ | 10 | 7 (5) | 3 (5) | | |
| $D_1D_2 \times$ | 82 | 36 (41) | 46 (41) | | |
| $D_1E \times$ | 5 | 4 (2.5) | 1 (2.5) | | |
| $AD_2 \times D_2D_2$ | 649 | 374 (324.5) | 275 (324.5) | | |
| $D_1D_2 \times$ | 1111 | 604 (555.5) | 507 (555.5) | | |
| $D_2E \times$ | 248 | 131 (124) | 117 (124) | | |
| $D_2E \times EE$ | 1 | 1 (0.5) | — (0.5) | | |
| Total | 2194 | 1202 (1097) | 992 (1097) | 20.10 | < 0.0001 |

Expected values are in parentheses.

**Table IV**

Expected and observed numbers of homozygotes and heterozygotes in matings where the "unlike-homozygous-mother effect" should not exist

| Matings ♂×♀ | N° | Homozygous progeny | Heterozygous progeny | $\chi^2$ | P (df.:1) |
|---|---|---|---|---|---|
| $AA \times AD_1$ | 9 | 6 4(.5) | 3 (4.5) | | |
| $\times AD_2$ | 48 | 23 (24) | 25 (24) | | |
| $\times AE$ | 2 | — (1) | 2 (1) | | |
| $AD_1 \times AD_1$ | 18 | 4 (9) | 14 (9) | | |
| $AD_2 \times AD_2$ | 429 | 227 (214.6) | 202 (214.5) | | |
| $AE \times AE$ | 3 | 1 (1.5) | 2 (1.5) | | |
| $D_1D_1 \times AD_1$ | 10 | 5 (5) | 5 (5) | | |
| $\times D_1D_2$ | 40 | 16 (20) | 24 (20) | | |
| $\times D_1F$ | 1 | 1 (0.5) | — (0.5) | | |
| $\times D_1E$ | 1 | — (0.5) | 1 (0.5) | | |
| $D_1D_2 \times D_1D_2$ | 649 | 324 (324.5) | 325 (324.5) | | |
| $D_1E \times D_1E$ | 5 | 3 (2.5) | 2 (2.5) | | |
| $D_2D_2 \times AD_2$ | 692 | 340 (346) | 352 (346) | | |
| $\times D_1D_2$ | 525 | 267 (262.5) | 258 (262.5) | | |
| $\times D_2E$ | 70 | 33 (35) | 37 (35) | | |
| $D_2E \times D_2E$ | 36 | 20 (18) | 16 (19) | | |
| Total | 2538 | 1270 (1269) | 1268 (1269) | 0.002 | 0.95 |

Expected values are in parentheses.

**Table V**

The analysis of the segregations of different matings involving the six transferrin alleles according to the method of KHATTAB *et al.* (1964)

| Matings | N° | AA | A+ | | ++ | $\chi^2$ | d.f. |
|---|---|---|---|---|---|---|---|
| AA×AA | 5 | 5 (5) | | | | | |
| AA×A+ | 59 | 29 (29.5) | 30 | (29.5) | | 0.02 | 1 |
| AA×++ | 84 | 84 | 84 | (84) | | | |
| ++×AA | 247 | | 247 | (247) | | | |
| A+×AA | 88 | 45 (44) | 43 | (44) | | 0.05 | 1 |
| A+×A+ | 707 | 157 (176.75) | 343 | (353.5) | 207 (176.75) | 7.68 | 2 |
| A+×++ | 1425 | | 624 | (712.5) | 801 (712.5) | 21.99 | 1 |
| ++×A+ | 2125 | | 1042 | (1062.5) | 1083 (1062.5) | 0.78 | 1 |
| | | $D_1D_1$ | $D_1$ | | ++ | | |
| $D_1D_1$+$D_1D_1$ | 5 | 5 (5) | | | | | |
| $D_1D_1$×$D_1$+ | 52 | 22 (26) | 30 | (26) | | 1.23 | 1 |
| $D_1D_1$×++ | 132 | | 132 | (132) | | | |
| ++×$D_1D_1$ | 146 | | 146 | (146) | | | |
| $D_1$+×$D_1D_1$ | 97 | 47 (48.5) | 50 | (48.5) | | 0.08 | 1 |
| $D_1$+×$D_1$+ | 998 | 227 (249.5) | 508 | (499) | 263 (249.5) | 2.93 | 2 |
| $D_1$+×++ | 2368 | | 1116 | (1184) | 1252 (1184) | 7.812 | 1 |
| ++×D+ | 1503 | | 756 | (751.5) | 747 (751.5) | 0,061 | 1 |
| | | $D_2D_2$ | $D_2$+ | | ++ | | |
| $D_2D_2$×$D_2D_2$ | 1124 | 1124 (1124) | | | | | |
| $D_2D_2$×$D_2$+ | 1287 | 640 (643.5) | 647 | (643.5) | | 0.04 | 1 |
| $D_2D_2$×++ | 424 | | 424 | (424) | | | |
| ++×$D_2D_2$ | 371 | | 371 | (371) | | | |
| $D_2$+×$D_2D_2$ | 2008 | 1109 (1004) | 899 | (1004) | | 21.96++ | 1 |
| $D_2$+×++ | 816 | | 431 | (408) | 385 (408) | 2.59 | 1 |
| ++×$D_2$+ | 436 | | 230 | (218) | 206 (218) | 1.32 | 1 |
| | | FF | F+ | | ++ | | |
| ++×F+ | 10 | | 3 | (5) | 7 (5) | 1.60 | 1 |
| | | EE | E+ | | ++ | | |
| ++×EE | 4 | | 4 | (4) | | | |
| E+×EE | 1 | 1 (0.5) | 0 | (0.5) | | | |
| E+×E+ | 65 | 11 (16.25) | 34 | (32.5) | 20 (16.25) | 2.63 | 2 |
| E+×++ | 860 | | 386 | (430) | 474 (430) | 9.04 | 1 |
| ++×E+ | 342 | | 186 | (171) | 156 (171) | 2.63 | 1 |
| | | $I_HI_H$ | $I_H$+ | | ++ | | |
| ++×$I_H$+ | 2 | | 1 | (1) | 1 (1) | | |
| | P < 0.05 | P < 0.01 | | | P < 0.001 | | |

zygous progeny; *i.e.*, in those matings where the bulls are heterozygous and the cows are homozygous for the same transferrin allele the observed number of the homozygous calves with the mother's phenotype is significantly higher than the number of heterozygotes with the phenotype of the father. There was no difference between the homozygous and heterozygous progeny ($P > > 0.95$) in the case of reciprocal matings, *i.e.*, where the bulls were homozygotes and the cows heterozygotes (Table IV).

The analysis of the segregations in different matings involving the six transferrin alleles by using the method of KHATTAB *et al.* (1964) also shows significant differences (Table V).

The segregation data of other matings between such parents, where the "unlike-homozygous-mother effect" should not exist, are interesting from the view-point of heterozygote superiority. ASHTON (1965) collected the segregation data of a lot of papers and found a significant difference between the number of homozygous and heterozygous progeny from these matings. The present data do not show this difference (Table IV). The excess of heterozygotes does not exist in our three populations (Table VI), *i.e.*, there are no significant deviations from the expected values calculated on the basis of the HARDY-WEINBERG's expectation.

**Table VI**

**The distribution of homozygous and heterozygous phenotypes in the three populations**

| Population of | Homozygotes | Heterozygotes |
|---|---|---|
| A. I. bulls | 219 (210.337) | 265 (273.663) |
| Cows | 4096 (4078.320) | 5070 (5087.680) |
| Progeny | 4133 (4129.416) | 5033 (5036.584) |
| Total | 8448 (8418.073) | 10368 (10 397.927) |
| | $x^2 = 0.182$ | $P = 0.70-0.60$ (df.: 1) |

Expected values are in parentheses.

## Discussion

EFREMOV *et al.* (1971) reported that the bovine serum transferrin is composed of two subunits in contrast to human transferrin which is a single polypeptide chain. WANG *et al.* (1966, 1967) found some of the electrophoretically different human transferrin variants to be different also in the substitution of an amino acid residue. These observations strongly suggest that the so-called maternal-foetal incompatibility (unlike-homozygous-mother effect) is caused by an immunological mechanism between the mother and the embryo.

The other three phenomena mentioned by ASHTON (1965) which were able to cause differences in the reproductive performance axing to the transferrin system are explainable by the findings of other authors, who demonstrated some physiological differences among the different transferrin variants, *e.g.*, in their iron-binding capacity (NEETHLING and OSTERHOFF, 1966) and on the superiority of the $Tf^E$ allele in adaptability (OSTERHOFF, 1966). However, some contradictory findings were also published by several authors. BUIS (1972) found no significant difference in the iron-binding capacity of the different transferrin variants. DOSTAL and HUNTER (1972) demonstrated that both the iron-binding capacity and the sexual-hormone-transporting capacity of the different transferrins *in vitro* are affected by some other factors independent of the transferrin phenotype. So it is strongly suggested that the reliable reasons for the deviation of the observed numbers from the expected values in the case of a large population are more immunological than physiological in nature. However, owing to the fundamental role of transferrin in the metabolism, the role of physiological factors cannot be excluded. On the other hand, the exact demonstration of the existence of such physiological effects would require further experimental verification.

## Summary

Transferrin gene frequencies were estimated and segregation data were collected on the basis of 9166 matings of 18,816 animals from the Hungarian Spotted (Fleckvieh) cattle breed. Six transferrin alleles were found. The matings between homozygous cows and heterozygous bulls showed a significant deficiency of heterozygous progeny, *i.e.*, the "unlike — homozygous — mother effect" (maternal — foetal incompatibility) was demonstrable. Furthermore, the segregation analyzis by the method of KHATTAB *et al.* (1964) showed also significant differences with regard to the segregation of phenotypes of the various alleles. Our data seem to confirm the assumption of other authors that the transferrin system of cattle is not a balanced polymorphism and it is probably affected by at present unknown immunological and/or physiological effects.

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Address of the authors: Dr. Péter SOOS, 8083, Csákvár, Berényi u. 3, dr. Erzsébet GIPPERT, Géza CSONTOS, 1144 Budapest, Remény u. 42, Hungary.

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# ON DIPLOSTOMOSIS OF THE GRASSCARP FRY

By

K. MOLNÁR

Veterinary Medical Research Institute (Director: D. DERZSY),
Hungarian Academy of Sciences, Budapest

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The diplostomosis of various fish species is well known and the life cycles of several *Diplostomum* species have been investigated in great detail, especially by SHIGIN (1964, 1968, 1969). The developmental cycle of *Diplostomum spathaceum* (Rud., 1819), most frequently occurring among fishes in Hungary, was followed up by CICHOWLAS (1961).

The pathogenic effect of *Diplostomum* parasites has been little studied. Several authors reported that the establishment of metacercariae in the lens caused blindness. Growth retardation by diplostomosis was observed by USPENSKAYA (1957) in the brown trout and by MINDEL (1963) in *Coregonus* sp. MINDEL also observed losses and so did EGELSKIY (1965) among infected sturgeon fry, which had just started to feed. In both cases, migrating larvae were responsible for the losses.

The development of the larval stages of *Diplostomum* species was studied by ERASMUS (1958, 1959) on fishes experimentally infected with *Cercaria*-X species from different *Lymnaeidae;* he investigated the mode of infection, cercaria migration within the host and the relationship between the counts of infective cercariae and losses. As ERASMUS did not precisely determine the species of the metacercariae establishing themselves in the lens, it can only be tentatively inferred that he was working with *Diplostomum spathaceum.*

*Diplostomum*-infestation has also been known among grasscarps *(Ctenopharyngodon idella)*, silver carps *(Hypophthalmichthys molitrix)* and marmor carps *(Hypophthalmichthys nobilis)* (AKHMEROV, 1963; SZAKOLCZAI and MOLNÁR, 1966; BAUER *et al.*, 1969; SHIGIN, 1971), but deaths from this parasitosis have not yet been observed. The investigations were focused on cataractic lesions of the lens in advanced diplostomosis. SHIGIN (1971) demonstrated that diplostomosis causes growth retardation also in grasscarp and AKHMEROV (1963) pointed out that *D. spathaceum*-metacercariae are pathogenic for herbivorous fishes, but presented no evidence.

In this paper an outbreak of diplostomosis which caused great losses among the grasscarp fry is reported. The circumstances of death, symptoms, gross lesions and a histological follow-up of larval migration are described in detail.

## Material and Methods

The examinations were conducted on 1.7—2.5 cm long, 3-week-old grasscarp fry procured from a pond farm. The young fishes were sectioned under a stereo-microscope and impression smears from different organs were investigated by light microscopy.

For histological examination, the fishes were fixed *in toto* in 4% formalin solution. The sections were cut longitudinally, so as to comprise the eye and were stained with haematoxylin and eosin or Farkas—Mallory's technique.

Species determinations were carried out according to SHIGIN (1968) on metacercariae collected from fingerlings died during the outbreak and later from survivors. In the first case, care was taken to use older stages, already showing the species characteristics.

## Results

In July 1972, an outbreak causing high losses occurred among the 3-week-old, 1.7—2.5 cm long grasscarp fry in a pond farm of Eastern Hungary. The fingerling pond in which the outbreak was observed, had been stocked with equal populations of newly-hatched grasscarp and silver carp fry some time ago.

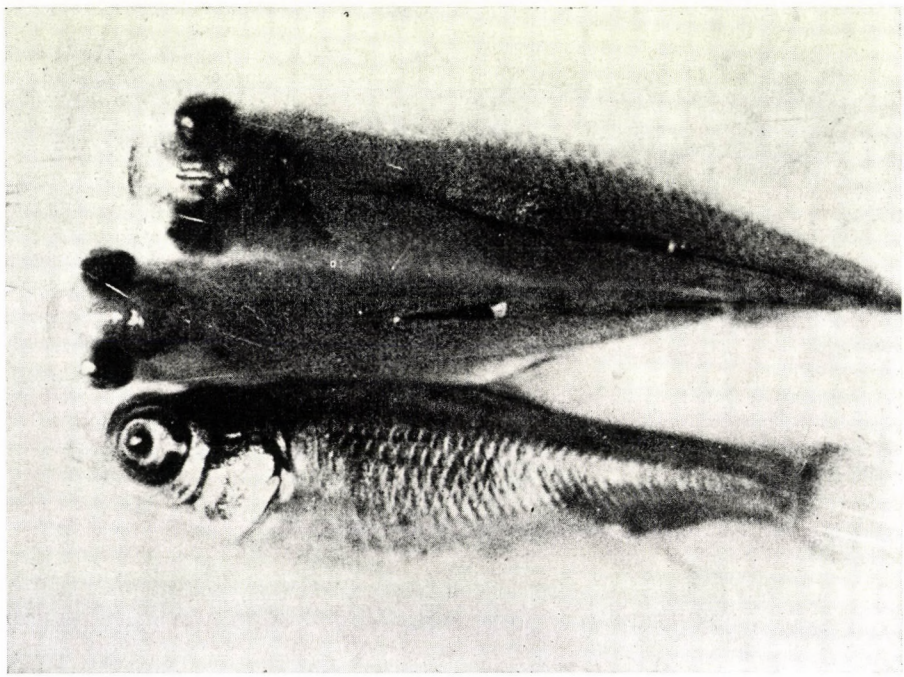

*Fig. 1.* Young 2.5 cm long grasscarp diseased in diplostomosis. Note protruding eyes and haemorrhages in the orbital cavity and skull area

Within a short time, 90% of the young grasscarps died of the disease, but no losses occurred among the silver carps, which were of the same age and size. The grasscarps taken from the pond for laboratory examination died in our aquaries within 48 hours, whereas all silver carps collected along with them survived.

In pond and aquary alike, the diseased fry showed characteristic symptoms. They did not respond to external stimuli and could easily be caught.

Some of them performed compulsive movements, usually in a rotating manner and the still weaker individuals floated on the water surface with the abdominal side upwards. The vivid red colour and congested blood vessels of the skull region were well-visible at first sight. The swollen eyeballs were nearly double of the normal size, protruded from the orbital cavity and were vivid red like the top of the skull (Fig. 1). Some specimens had already lost one eye. The opaque, often milky white lens was scarcely visible because of haemorrhages in the ocular chamber.

### *Parasitological section*

Many *Diplostomum* larvae, in different stages of development, were found in the lens. Migrating *Diplostomum* cercariae occurred in the vitreous body and other parts of the eye as well as in the brain, heart and muscles. The migrating stages were smaller than those which established themselves in the lens and, unlike metacercariae, they had a well-visible gut and ventral sucker; the suckers and integument were covered by spines. Most larval stages (20—25 per host) localized in the lens, but relatively many (4—5 per host) were present elsewhere in the eye. The average larval number in other body regions was 7—10 migrating stages per host. Except for some *Trichodina* on the gills, no other parasite species was found.

The silver carps were infested by *Diplostomum* to a similar degree as grasscarps and they also had coccidiosis, but neither symptoms nor losses occurred among them. Grasscarps from four adjacent fingerling ponds of the same farm were examined as controls; these had a much lower degree of metacercarial infestation, harbouring on the average 5—15 parasites per host. Certain severely infested individuals of the control lot showed, nevertheless, the same symptoms as described above.

### *Examination on metacercariae*

Among the metacercariae collected from the infected fry and other grasscarps all stages which already had shed the larval integument showed the features described by SHIGIN (1968) as characteristic of *Diplostomum spathaceum* (Rud., 1819).

### *Histological examinations*

The metacercariae which established themselves in the lens, above all at its periphery (Fig. 2), were easily demonstrable histologically. They caused slight lesions: an epithelial proliferation was only seen at the site of larval penetration. Cercariae migrating towards the lens were found in different parts

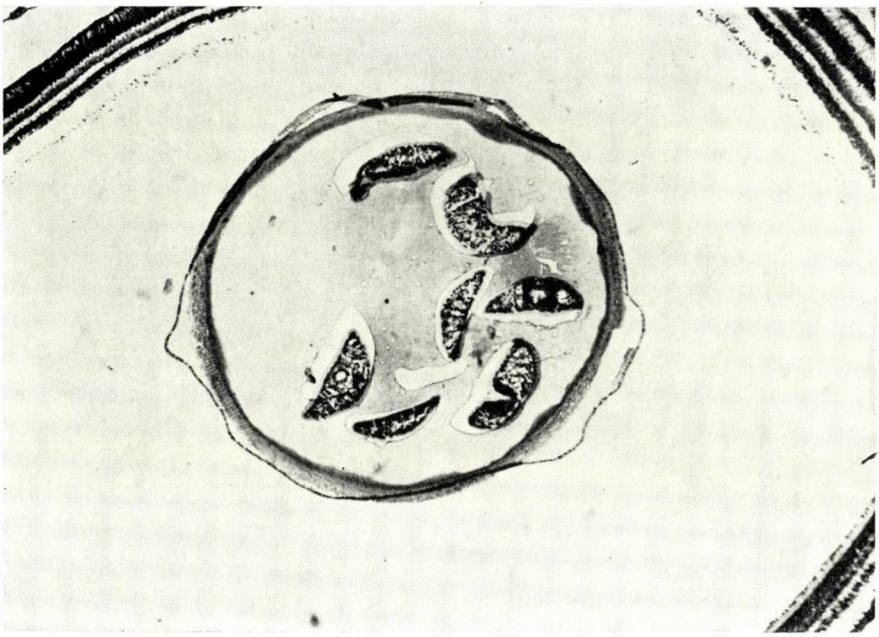

*Fig. 2.* Young metacercariae established in the lens. Haematoxylin and eosin staining, ×200



*Fig. 3. Diplostomum*-cercaria surrounded by proliferative tissue close to the pigmented chorioidea

of the eye, in the cornea, retina and vitreous body, surrounded by cells characteristic of the localization and by blood corpuscles. In certain cases, however, the cercariae were surrounded by granulation tissue (Fig. 3), occurring above all in the orbital cavity, but also in the periocular connective tissue and inside




*Fig. 4.* Tissular reaction around a cercaria established in the ocular cavity of the grasscarp. *a*, Cercaria in the vitreous body; *b*, haemorrhages in the vitreous body; *c*, transfixed cercaria in the retina; *d*, cercaria surrounded by proliferative tissue in the chorioid. Haematoxylin and eosin stain, × about 60

the eyeball. Granulation was most frequently seen around the larvae localizing in the chorioid body, but it also occurred in the retina and vitreous body (Fig. 4). No such tissular reaction was seen around larvae migrating in other body regions, e.g. in the heart (Fig. 5) and skull area (Fig. 6). In addition to tissular reactions, there were extensive haemorrhages in the eye chamber, vitreous body, chorioid and cerebral meninx (Fig. 7).



*Fig. 5.* Heart of grasscarp in longitudinal section. Note the cross-section of cercaria between auricle and ventricle

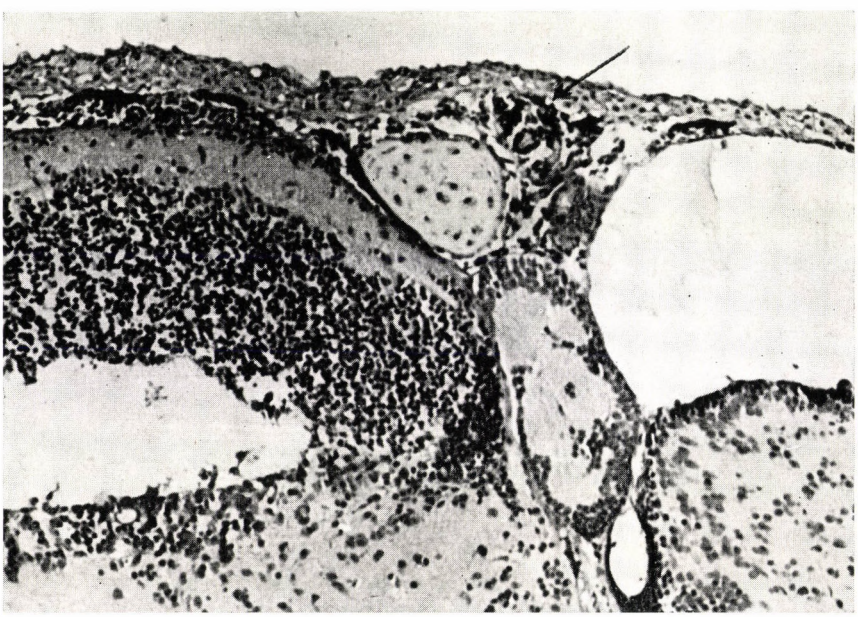

*Fig. 6.* Skull area of grasscarp. Note the penetrating cercaria under the epithelium

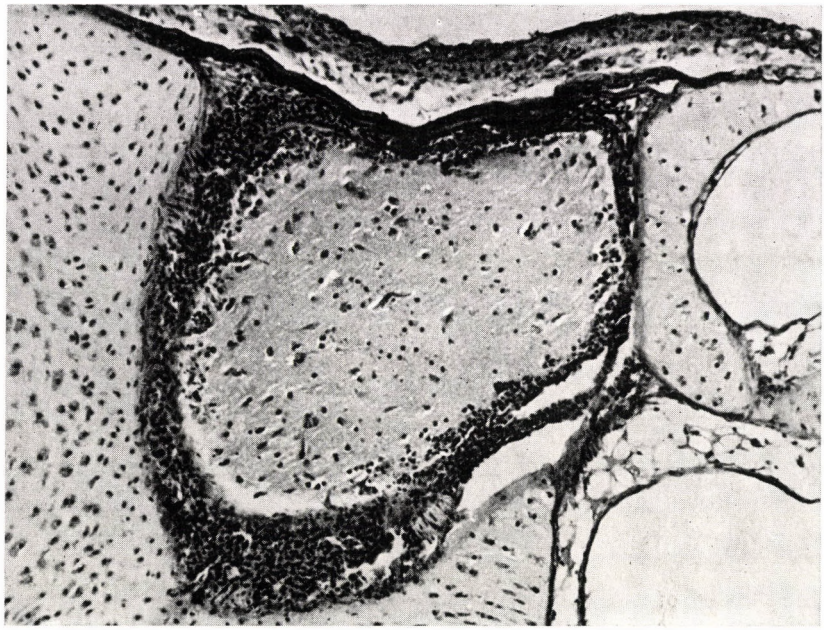

*Fig. 7.* Haemorrhages in the meninx of a grasscarp

## Discussion

It was reported earlier (SZAKOLCZAI and MOLNÁR, 1966) that in Hungary, the herbivorous fishes were much more extensively infested with *Diplostomum spathaceum* metacercariae than the common carp and that 5—8 cm long, one-summer grasscarps were not infrequently found to harbour 200—300 metacercariae in the ocular lens. The damages of metacercaria-diplostomosis could not be estimated because *Diplostomum* infestation is so frequent among grasscarps that no appropriate controls were available. Thus we were unable to clarify the relationship of the growth retardation described by USPENSKAYA (1957), MINDEL (1963) and SHIGIN (1971) with the diplostomosis-associated lenticular cataract, because this was present in practically all grasscarps.

As in the outbreak studied, other parasitoses and a dietary failure could be excluded, it seems certain that it was due to a cercaria-diplostomosis, *viz.*, the losses were caused by migrating *Diplostomum spathaceum* larvae. The fatal outcome depended on the number of larvae establishing themselves in, and migrating in, the body of the host: 20—25 sessile and 10—15 migrating diplostomulum stages killed the 1.7—2.5 cm long young grasscarps.

The lethal changes already develop during migration and the larvae establishing themselves in the lens do not seem to play a decisive role. This is in good accordance with the observation that in a similar outbreak among

common carps in the previous year the hosts did not show eye symptoms and harboured only 1—2 parasites, if any, in the lens, but migrating *Diplostomum* larvae were always present in their body. In hosts showing compulsive movements, the migrating stages were chiefly found in the brain and spinal cord.

In the outbreak studied, the grasscarps showed the characteristic symptoms of cercaria-diplostomosis, corresponding essentially with those described by PETRUSHEVSKIY and BAUER (1953) .

We do not agree with MUSSELIUS (1967) who established that the grasscarp is less susceptible to *Diplostomum* infestation than the silver or marmor carps. The apparent contradiction can probably be ascribed to the dissimilar water levels of fingerling ponds in the USSR and Hungary. In this country, they are generally shallow, not allowing for the separation of the biotopes of grasscarp, silver carp and marmor carps, whence the chances of cercaria-infestation are equal for all the three species. My own experience is that when grasscarp and silver carp fry of the same age and size were reared together in one and the same fingerling pond, the former proved to be more susceptible to migrating larvae.

The severe outbreak of diplostomosis which involved the entire grasscarp population of the fingerling pond can be regarded as a warning that cercaria-infestation may be more frequent than inferred from reported cases. Unexplicable "disappearances" of fry from the pond may not infrequently have been due to this disease.

The results of histological examinations correspond with the findings of ERASMUS (1959) and suggest that cercariae can penetrate the body of the host at several sites. The occurrence of migrating cercariae in different tissues of the body also supports ERASMUS's opinion that the blood stream plays little role in larval migration towards the eye.

The route of the cercariae is marked by extensive haemorrhages, above all in the richly vascularized areas, such as the cerebral meninges and orbital cavity. Death occurs when the cercariae, being relatively large compared to the dimensions of the host, injure vitally important organs by causing haemorrhage or otherwise. In this respect the meningeal haemorrhages are more deleterious than even the severest ocular lesions.

A fatal outcome of diplostomosis seems to occur when a very young host which had not previously been infected is heavily invaded by cercariae.

The spawning of herbivorous fishes requires a relatively high temperature and thus takes place late in the season. Consequently, the rearing of the fry coincides with the appearance of the factors mediating parasite invasion, *viz.*, snails and young birds. Exposure to *Diplostomum* infestation is therefore greater in the case of herbivorous fishes than for other cultured fishes.

It was shown histologically that part of the penetrating parasites elicit a special reaction, characterized by tissue proliferation. Proliferative tissue

was usually found within the eyeball or in its immediate surroundings. It is remarkable that the lens, the site of preference, showed only slight lesions: proliferation of the lenticular epithelium was only seen at the sites of penetration, as already observed by MAWDESLEY-THOMAS (1972).

The direct causes of tissue proliferation are not yet fully understood. It might represent the protective mechanism of the host or, as suggested by ERASMUS (1959), simply the encapsulation of cercariae having lost viability during migration. Thirdly, it may be a tissular reaction to the direct mechanical effect of the established stages or to the chemical effect of their metabolic and other products.

## Summary

Infestation by cercariae of *Diplostomum spathaceum* killed 90% of the 3-week-old, 1.7—2.5 cm long grasscarp *(Ctenopharyngodon idella)* fry reared in a fingerling pond. The severely diseased hosts harboured on the average 20—25 larvae in the ocular lens and 10—15 migrating stages in other parts of the body.

Cercarial migration caused haemorrhages, visible even externally.

Most cercariae which established themselves in the eye or the adjacent areas were surrounded by proliferative tissue.

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Address of the author: Dr. Kálmán MOLNÁR, 1143 Budapest, Hungária krt. 21, Hungary

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# CONTRIBUTION TO THE HAEMOGRAMS OF THE BUFFALO AND OF THE CAMEL

By

J. Bokori

UNDP/FAO Near East Animal Health Institute, Iraq
Unit and College of Veterinary Medicine, Abu Ghraib, Baghdad, Iraq

(Received March 19, 1973)

Information is scanty on the haemogram of the buffalo and, especially, on that of the camel, even in the fundamental textbooks. The available data are incomplete, sometimes even controversial, with little experimental background. This can on the one hand be ascribed to the fact that large herds of these animals are chiefly kept in developing countries in which the main veterinary concern is still the control of infectious and parasitic diseases, on the other hand to the difficulty of obtaining blood samples from these animals.

A brief survey of the available haematological data of the two species is forthcoming. Hafez and Anwar (1954) gave the average erythrocyte and leucocyte counts of the buffalo as 6.8 million and 6.7 thousand, respectively, its haemoglobin level as 13% and its haematocrit value as 44.3%. The differential count was 51% lymphocytes, 8% monocytes (?), 36.0% neutrophils, 5% eosinophils and 1% basophils. Erythrocyte sedimentation rate (ESR) was 2—8 mm/hr according to Hafez and Anwar and 55—60 mm/hr according to Purushotham and Mahendar (1963). David L. Coffin (1957) determined the erythrocyte count of the buffalo as 5.4—7.4 million and the average haemoglobin content as 11.8 g %.

The haemogram of the camel was studied by Soni and Aggarwala (1958) and Banerjee *et al.* (1962), who give the average values of erythrocyte count as 8.2 and 7.24 million, leucocyte count as 20,100 and 18,100, haemoglobin as 15.5 and 13.1%, respectively, and the haematocrit value uniformly as 27%. As to the differential count, Soni and Aggarwala regarded the lymphocytes (46%), Banerjee *et al.* the neutrophils (50.6%) as the major component.

Wirth (1931) found on the average 9.3 million erythrocytes, 10,150 leucocytes and 11.83 % haemoglobin, and described the differential count as 55% lymphocytes (?), 6% monocytes, 35% neutrophils, 2.5% eosinophils and 0.5% basophils. The diameters of the elliptic red blood cells were determined by Wirth as $7.35 \times 4$ $\mu$m, by David L. Coffin (1957) and Kohli (1963) as $7.5 \times 4.2$ $\mu$m. Banerjee *et al.* found an ESR of 1 mm/hr. and Bhatt and Kohli (1959) found only a few mm after 7 hrs.

## Materials and Methods

Blood samples were taken from 20 healthy and 8 diseased adult buffaloes, from 12 healthy and 7 diseased young (1—11 months old) buffaloes and from 35 camels sent to the abattoir for slaughter. The samples were withdrawn

* This paper was prepared while the author was serving on a project of the Government of Iraq, financed by the United Nations Development Programme and executed by the Food and Agriculture Organization of the United Nations.

from the jugular vein, from the camels usually during exsanguination. EDTA was added to each sample to prevent clotting and the samples were transported to the laboratory in plastic coolers for immediate processing.

Erythrocyte and leucocyte counts were determined in Neubauer's haemocytometer, after an appropriate dilution. The haematocrit value was determined by means of Hawskley's micro-haematocrit centrifuge.

Haemoglobin was determined in Spencer's haemoglobinometer. Smears prepared at sampling or immediately afterwards were stained according to the technique of Giemsa or Leishman, and 100 cells were counted on each slide for evaluation.

ESR was determined according to Westergreen, using dilutions of 1 : 4 in a 3.8% Na-citrate solution. It was read at 15, 30, 60 and 120 min and at 24 hrs with buffalo blood and at 1, 2, 24, 48, 72 hrs, occasionally also at 96 hrs, with the slowly sedimenting camel blood. Erythrocyte dimensions were determined by means of an ocular micrometer.

## Results and Conclusions

The limit and average values for healthy buffaloes and individual data of diseased young and adult buffaloes are shown in Table I, extreme and average values for camels in Table II.

Erythrocyte counts ranged from 4.1 to 6.7 million/$\mu$l (average: 5.8 million) in healthy adult buffaloes and from 4.2 to 8.4 million (average: 6.5 million) in healthy young ones. Thus the young animals had slightly more red blood cells than the older ones, but the average values for both were slightly less than the 6.8 million reported by HAFEZ and ANWAR (1954). Diseased buffaloes, either young (5.5 million) or adult (4.5 million), had lower average erythrocyte counts than the healthy ones.

Mean leucocyte counts did not notably differ between healthy young and adult animals. These values slightly exceeded the average count of 6,700 given by HAFEZ and ANWAR (1954). In young and adult buffaloes diseased in different conditions, the mean leucocyte count rose by about 50% above the normal level (12,400 and 11,700, respectively).

The haemoglobin level of blood from healthy adult animals ranged between 10.5 and 14.5%, those of buffalo calves from 10.7 to 14.6%, averaging 12.3 and 12.8%, respectively.

The haematocrit values ranged from 28 to 38 and 27 to 40% on the average. HAFEZ and ANWAR (1954) established the mean haemoglobin level for 20 buffaloes as 13%, the haematocrit value as 44.3%. DAVID L. COFFIN (1957) determined the haemoglobin value as 11.8%.

The haemoglobin and haematocrit values of diseased buffaloes were only slightly below the normal.

**Table I**

Haemograms for healthy and diseased buffaloes and buffalo calves

| No. | Age of animals | Clinical picture | Red blood cells. Count million/µl | | Hb % | Htc % | White blood cells. Count thousand/µl | Differential count | | | | | Erythrocyte sedimentation rate mm/hour | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Lympho-cytes % | Mono-cytes % | Neutro-phils % | Eosino-phils % | Basophils % | 1/4 | 1/2 | 1 | 2 | 24 |
| 20 | 2—9 years | Healthy animals | Limits | 4.1—6.7 | 10.5—14.5 | 28—38 | 5.20—12.70 | 40—64 | 0—2 | 28—50 | 2—23 | 0—1 | 1—7 | 1.5—27 | 2.5—62 | 7—92 | 100—138 |
| | | | Average | 5.8 ±1.07 | 12.3 ±1,13 | 32.4 ±3.1 | 8.25 ±1.84 | 50.5 ±5.6 | 1.0 ±0.3 | 40.0 ±7.4 | 8.5 ±5.7 | 0.3 ±0.48 | 3.2 ±2.0 | 8.4 ±8.24 | 26 ±24 | 60 ±24 | 121 ±12.7 |
| | | Traumatic reticulo-peritonitis | | 4.8 | 13.4 | 36 | 10.7 | 13 | 1.0 | 85 | 1.0 | 0 | 11 | 29 | 72 | 110 | 135 |
| | | Anaemia | | 2.6 | 9.1 | 21 | 6.6 | 33 | 1.0 | 63 | 3.0 | 0 | 10 | 27 | 68 | — | 129 |
| | | Metritis | | 5.6 | 12.1 | 34 | 8.1 | 43 | 1.0 | 54 | 2.0 | 0 | 14 | 40 | 80 | 112 | 135 |
| | | Squamous cell carcinoma of eyelid | | 4.2 | 13.0 | 29 | 10.0 | 40 | 2.0 | 49 | 9.0 | 0 | 40 | 80 | 130 | 138 | 140 |
| | | Prolapse of the uterus | | 4.8 | 12.5 | 33 | 12.5 | 41 | 0.0 | 58 | 1.0 | 0 | 5 | 7 | 56 | 84 | 132 |
| | | Suspicion of peritonitis | | 4.6<br>4.5 | 12.9<br>11.9 | 32<br>30 | 13.2<br>14.1 | 34<br>19 | 0.0<br>0.0 | 65<br>80 | 1.0<br>1.0 | 0<br>0 | 10<br>12 | 25<br>29 | 65<br>70 | 100<br>110 | 131<br>133 |
| | | Ruminal fistula | | 4.9 | 12.8 | 34 | 13.3 | 24 | 1.0 | 74 | 1.0 | 0 | 10 | 25 | 50 | 80 | 125 |
| 8 | 4—12 years | | Limits | 2.6—5.6 | 9.1—13.4 | 21—36 | 6.6—14.1 | 13—43 | 0.0—2.0 | 49—85 | 1.0—9.0 | 0 | 5.5—40 | 5—40 | 50—130 | 80—138 | 125—140 |
| | | | Average | 4.5 ±0.87 | 12.2 ±1.35 | 31.1 ±4.7 | 11.7 ±3.3 | 31 ±11.1 | 0.7 ±0.5 | 66 ±12.7 | 2.3 ±2.8 | 0 0 | 14 ±10.5 | 32.7 ±21.1 | 74 ±24.5 | 105 ±19.5 | 133 ±4.5 |
| 12 | 1—7 years | Healthy animals | Limits | 4.2—8.4 | 10.7—14.6 | 27—40 | 6.15—11.40 | 38—72 | 0.0—1.0 | 26—50 | 1.0—11.0 | 0—1 | 1—6 | 3—18 | 6—38 | 12—58 | 76—124 |
| | | | Average | 6.5 ±1.76 | 12.8 ±1.31 | 34.1 ±4.2 | 8.59 ±1.71 | 57 ±9.1 | 0.5 ±0.5 | 39 ±7.2 | 3.5 ±3.3 | 0.09 ±0.04 | 3.2 ±1.75 | 9.8 ±5.88 | 19 ±12.3 | 28.7 ±16.1 | 101 ±20.7 |
| | | Pneumonia | | 3.5 | 10.7 | 26 | 17.4 | 26 | 1.0 | 70 | 3.0 | 0 | 10 | 28 | 70 | 98 | 140 |
| | | Enteritis | | 6.3 | 13.2 | 34 | 11.3 | 42 | 1.0 | 54 | 3.0 | 0 | 2 | 5 | 11 | 17 | 98 |
| | | Keratitis | | 6.8 | 14.0 | 35 | 10.1 | 33 | 1.0 | 65 | 1.0 | 0 | 2 | 5 | 18 | 38 | 88 |
| | | Haemolytic icterus | | 5.7 | 14.2 | 36 | 10.0 | 42 | 1.0 | 56 | 1.0 | 0 | 7 | 15 | 33 | 66 | 130 |
| | | Chronic catarrhal pneumonia | | 4.7 | 8.0 | 38 | 18.0 | 38 | 1.0 | 59 | 2.0 | 0 | 8 | 15 | 40 | 72 | 145 |
| | | Encephalitis | | 6.2 | 11.6 | 32 | 10.3 | 47 | 1.0 | 50 | 2.0 | 0 | 4 | 9 | 24 | 68 | 120 |
| | | Lung helminthosis | | 5.2 | 10.7 | 30 | 9.8 | 47 | 1.0 | 44 | 8.0 | 0 | 3 | 7 | 20 | 70 | 129 |
| 7 | 4—11 months | | Limits | 3.5—6.8 | 8.0—14.2 | 26—38 | 9.8—18.0 | 26—47 | 1.0—1.0 | 44—70 | 1.0—8.0 | 0 | 2—10 | 5—28 | 11—70 | 17—98 | 88—145 |
| | | | Average | 5.5 ±1.0 | 11.8 ±2.21 | 31.1 ±3.8 | 12.4 ±3.66 | 39 ±7.7 | 1.0 — | 57 ±8.8 | 3.0 ±2.4 | 0 — | 5 ±3.1 | 12 ±8.2 | 31 ±19.8 | 61 ±26.2 | 121 ±21.2 |

**Table II**

Haemograms of adult camels

| No. of | Age of camels | Red blood cells Count million/µl | | Hb g% | Htc % | White blood cells Count thousand/µl | Differential count | | | | | Erythrocyte sedimentation rate mm/hr | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Lymphocytes % | Monocytes % | Neutrophils % | Eosinophils % | Basophils % | 1/2 | 1 | 2 | 48 | 48 | 72 |
| 35 | 1.5—15 years | Limits | 3.75—8.10 | 8.6—14.4 | 24—39 | 7.8—30.0 | 31—80 | 0—1 | 18—66 | 1—20 | 0—2 | 0 | 0—2.5 | 1—5 | 8—70 | 18—96 | 60—131 |
| | | Average | 5.60 ±1.23 | 11.8 ±1.5 | 29.7 ±3.6 | 13.2 ±4.5 | 49.1 ±12.9 | 0.5— ±0.5 | 45.7 ±12.9 | 4.7 ±4.3 | 0.9 ±0.4 | 0 | 0.87 ±0.56 | 1.9 ±1.0 | 30.2 ±3.9 | 55.8 ±32.0 | 92 ±19 |

On the basis of the differential count, buffalo blood should be regarded as slightly lymphocytic. The mean percentage of lymphocytes was 50.5% in adults and 57% in calves. Diseased buffaloes, both young and adult, showed a considerable shift to the left of the differential count, the respective mean percentages of neutrophils having been higher than 57 and 66%. The mean percentages for eosinophils in healthy adult and young buffaloes were 8.5 and 3.5%, respectively.

Unlike other ruminants, buffaloes had surprisingly high ESR even normally. Values determined at 15, 30, 60, 120 min and at 24 hrs were 3.2, 8.4, 26, 60 and 121 mm, respectively, the latter being practically the final value. The values measured after one hour were considerably higher than those established by HAFEZ and ANWAR (1954) (2—8 mm), but reached only about one half of those reported by PURUSHOTHAM and MAHENDAR (1963) (55—60 mm).

A considerable low ESR was chiefly observed in adult diseased animals, especially during the first two hours. The mean values established at the above time intervals were 14, 32.7, 74, 105 and 133 mm.

Erythrocyte diameters ranged from 4 to 6.8 $\mu$m (average: 5.1 $\mu$m).

The 35 adult camels had mean erythrocyte and leucocyte counts of 5.6 million and 13,200, respectively, as calculated for one $\mu$l blood. The mean haemoglobin and haematocrit values were 11.8% and 29.7%.

Differential counting showed on average 49% lymphocytes, 0.5% monocytes, 45.7% neutrophils and 4.7% eosinophils. Basophils were only seen on 3 slides. Fourteen camels had more neutrophils than lymphocytes; the rest had a slightly lymphocytic blood picture. This accords well with the finding of SONI and AGGARWALA (1958) and BANERJEE et al. (1962) that camel blood may be slightly lymphocytic or neutrophilic.

It is known that the ESR of camels is very low. Practically no sedimentation occurred in the Westergreen tubes within 30 minutes. Mean values after 1, 2, 24, 48, 72 and 96 hours were 0.87, 1.9, 30.2, 55.8, 92 and 109 mm. Sedimentation was found to be diphasic (the borderline between settling erythrocytes and blood plasma was indefinite) in nearly half of the cases, whereas, in the rest the plasma and red cells were sharply demarcated.

## Summary

The haematological data (erythrocyte count, leucocyte count, haemoglobin, haematocrit, differential count and erythrocyte sedimentation rate; ESR) of a total of 47 adult and young, healthy and diseased buffaloes and of 35 adult camels are described. Comparison of the results according to age group and health state and with the few literary data available showed that both young and adult diseased buffaloes had fewer erythrocytes (5.5 and 4.5 million/$\mu$l, respectively) than their healthy age mates (6.5 and 5.8 million, respectively), but their leucocyte counts rose by about 50% (12,400 and 11,700) compared to normal. The haemoglobin and haematocrit values did not notably differ between healthy and diseased buffaloes. ESRs of the diseased animals were considerably higher, especially during the first two hours. Mean red blood cell diameter was 5.1 $\mu$m.

Camels had 5.6 million erythrocytes and 13,200 leukocytes/cu. mm blood; their haemoglobin and haematocrit values were 11.8 g% and 29.9%. The blood picture was either slightly lymphocytic or slightly neutrophilic. The ESR was extremely low; sedimentation lasted 72—96 hrs and was often diphasic from 24 hrs.

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The recent address of the author: Dr. József BOKORI, 1078 Budapest, Landler J. u. 2, Hungary.

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# THE GENERAL FEBRILE RESPIRATORY FORM OF INFECTIOUS BOVINE RHINOTRACHEITIS IN CALVES

By

A. BARTHA, J. KISARY and S. BELÁK

Institute of Epizootology and Bacteriology (Director: J. MÉSZÁROS), University of Veterinary Science, Budapest

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Infectious bovine rhinotracheitis (IBR) virus may cause in cattle clinically, epizootologically and pathologically distinct forms of disease, which differ from one another so markedly that prior to the identification of the common causal agent they had been regarded as independent conditions.

Out of them viral vaginitis (old term: infectious pustular vulvovaginitis) has been longest known; subsequently an infectious upper respiratory disease of above 6 months old beef cattle and dairy cows, known earlier as "red nose disease" or "dust pneumonia" was described in 1954 and termed on the suggestion of MCKERCHER and MOULTON (1957) infectious bovine rhinotracheitis (IBR). The aetiological identity of the two pictures was confirmed by GILLESPIE *et al.* in 1957, and since then the causal agent has been termed IBR virus or IBR-IPV virus. Soon afterwards it was disclosed that the virus can also cause mass incidence of a benign conjunctivitis, accompanied by no other symptoms, in cattle herds (ABINANTI and PLUMMER, 1961). A rare sporadic occurrence of lethal encephalitis among less than 5 months old calves (FRENCH, 1962) and, occasionally, abortion of cows (MCKERCHER and WADA, 1964), both due to IBR virus, were then reported.

Of the five pictures outlined in the foregoing, genuine infectious rhinotracheitis (DOMÁN and FODOR, 1961), viral vaginitis (MANNINGER *et al.*, 1962), calf encephalitis (BARTHA *et al.*, 1969) and conjunctivitis (JUHÁSZ, 1970) have been observed in Hungary. Abortion confirmed by virus isolation has not been reported. Recently, however, an IBR virus disease, distinct from all mentioned pictures, has occurred with increasing frequency in large cattle herds. The disease chiefly affects rearing calves and is manifested as a febrile condition with general symptoms. Up to now it has caused greater losses than the earlier known five pictures of IBR virus infection.

This paper is a report of observations on the new disease, which occurs in the form of epidemic-like outbreaks and causes great losses in large-scale calf herds.

## Clinical observations

*a)* In a co-operative farm calves originating from a dairy herd of 220 cows had been reared in isolation until 10 days of age, after which they were transferred to the rearing house and maintained under good conditions of management. In March, 1968, an acute respiratory disease, accompanied

by temperature elevation to 40.5—42 °C set in first in the 3-month-old age group (90 calves), involving all except the less than one-month-old animals within 2 weeks.

Eighty per cent of the 1—6 month-old calves became clinically ill, the remaining 20% showed no symptoms. Besides fever, the initial symptoms were listlessness, inappetence, serous nasal discharge, reddening of the nose and muzzle; some animals also showed foamy salivation and a mild diarrhoea. No ulceration occurred in the nasal cavity and mouth. The breathing was quick and difficult and, there was, chiefly on movement, a painful, dry cough. After 3—4 days the temperature normalized, respiratory symptoms ceased, the muzzle epithelium became detached in the form of a parchment-like sheet, but dyspnoea and dry cough persisted in about one third of the animals. In these cases the serous nasal discharge became first mucinous, then purulent, the dyspnoea developed more severe and the majority of the animals died with, or had to be emergency-slaughtered for, symptoms of a catarrhal-purulent pneumonia within 1—4 weeks. No losses occurred during the initial acute febrile course, and only a single calf had to be emergency-slaughtered. In the advanced stage, however, 19 calves were lost because of the catarrhal-purulent pneumonia. The calf emergency-slaughtered in the acute stage showed at post mortem examination a severe pulmonary emphysema and had three infiltrated foci of the size of a walnut in the peribronchial area. The bronchi contained a reddish, foamy exudation. The mucous membranes of the nasal cavity exhibited even reddening and superficial ulcerations, which also occurred on the tracheal mucosa. Calves died in the chronic stage of the disease showed, apart from a slight pulmonary emphysema, an extensive catarrhal-purulent inflammation of the lungs. Ulceration of the upper respiratory tract was absent even at this stage. No gross lesions were found in organs other than respiratory.

The transfer of newborn calves to the infected rearing house was continued after the outbreak, for lack of housing capacity. The young animals developed the symptoms of the disease 10—20 days after transfer. The disease did not spread to the herds of growing cattle and dairy cows.

To clarify the aetiology, pathological exudations from diseased animals and organs of carcases or emergency-slaughtered calves were submitted to the laboratory for virus isolation and blood samples were collected for virus-neutralization tests.

*b)* In another cooperative farm, 70 calves originating from the farm's own cow herd and 40 calves procured from elsewhere were maintained in the rearing house. Calves had regularly been purchased from other farms also earlier, but no notable disease occurred among the rearing animals until February, 1968, when one month old calves began to show the same non-characteristic general respiratory symptoms as observed in the other farm.

The disease involved all age groups within 3 weeks, including the 2—3 weeks old calves and later 4—5 days after transfer to the rearing house, even the youngest animals became ill. The symptoms were most severe among the less than 2 months old calves, but only one of them died during the acute stage. The majority of the calves developed a catarrhal-purulent pneumonia after the acute stage and losses from death or emergency slaughter amounted to 21%. Farm calves and purchased calves were equally affected. The disease did not spread to the herds of growing cattle and dairy cows.

*c)* The cattle herd of a third cooperative farm consisted of 300 cows reared in the farm and their offspring, and 80 pregnant heifers were purchased for replenishment. The latter began to show respiratory symptoms 3 weeks after their establishment with the herd. About half of the pregnant heifers developed temperature up to 41 °C, severe dyspnoea, serous nasal discharge, lacrimation, foamy salivation and a frequent, non-painful, moist cough. The disease spread to the cows reared in the farm, causing symptoms in about 10% of the group. Especially the first-pregnant heifers in advanced state were severely affected: one died and two had to be emergency-slaughtered. The other diseased animals recovered completely in 3—6 months and there was no notable reduction in milk production.

Post mortem examination of the spontaneously died or emergency-slaughtered calves revealed an acute extensive pulmonary emphysema and many petechial haemorrhages on the tracheal mucosa. No ulcerations or erosions were found either on the purple-red mucosa of the nasal cavity or on the tracheal mucosa. No signs of inflammation were found in the lungs.

Two weeks after the outbreak had ceased among the pregnant heifers and cows, the respiratory disease reappeared among fattener bulls and rearing calves. Ninety of the 100 fatteners developed an essentially benign respiratory disease, with non-characteristic general symptoms, such as temperature rise to 41—41.5 °C, difficult breathing, serous nasal discharge, lacrimation and, occasionally, foamy salivation and a frequent painful, dry cough. Only a single fattener bull had to be emergency-slaughtered for severe illness, the rest recovered within one week. There was no pneumonial complication, but the animals lost considerable weight during their illness.

The rearing calves, all originating from the cow herd of the farm, began to show the non-characteristic respiratory symptoms simultaneously with the outbreak in the fattener herd. The disease was in every respect similar to that observed in the first two farms. The one-month-old age group was affected first, then all age groups became involved within two weeks. Only 50% of the rearing calves showed symptoms, which were most severe among the 2—3 months old animals. No loss occurred during the acute stage, but later pneumonia and complications caused the death of 14 calves, spontaneously or by emergency slaughter, within 6 weeks.

Samples of exudation from diseased animals and organs from carcases were collected for virus isolation and paired serum samples were withdrawn from diseased calves, fattener bulls and cows for demonstration of virus neutralizing antibodies.

Virus isolations and serological tests unequivocally proved the responsibility of IBR virus for the outbreaks in all three farms.

## Materials and Methods

Nasal and conjunctival swabs from diseased animals were transported to the laboratory in melting ice. Specimens of lung, pulmonary lymph node, trachea, nasal mucosa, tonsil, spleen and liver from carcases were also transported under cooling. All test materials were processed within 24 hours.

The swabs were soaked and pressed in 2—3 ml Hanks' or PBS solution, the organ specimens were homogenized by grinding after addition of ten volumes of Hanks' solution containing penicillin and streptomycin. The organ homogenates were centrifuged twice for 15 minutes at 3000 r.p.m., and 0.1 ml amounts of each supernatant were inoculated into 4 calf kidney and 4 calf testicle cell cultures. The cell cultures were prepared by the technique employed in our institute, using Hanks' solution as maintenance medium containing 0.5% lactalbumin hydrolysate. The inoculated cultures were incubated for one week at 37 °C, the cytopathic changes occurring in the meantime were followed up daily by low-power microscopic examination. Each culture was carried through 5—10 blind passages, set up at one-week intervals. If cytopathic effect (CPE) has appeared, the passage was performed immediately.

The CPE was studied in greater detail in cultures stained with haemalaun and eosin. The isolate was identified by neutralization test, using rabbit antisera and 100 $TCID_{50}$ virus.

## Results

Only 9 virus strains were isolated from 138 samples originating from cattle showing the respiratory symptoms. All the isolates were obtained from acute cases; no virus could be isolated from animals with pneumonia. Isolations were also negative from cows (Table I).

Four isolates developed the specific CPE already in the first passage, the other five isolates in the second passage. Syncytium formation, granulation and condensation of chromatin along the nuclear membrane and Cowdry type A nuclear inclusions appeared in the first passage 3—4 days, in further passages after 1—2 days, after inoculation. The isolate proved to be chloroform-sensitive. On the basis of these properties, the isolates seemed to belong to the *Herpesvirus* group. This was confirmed by the subsequent serological tests, *viz.*, exclusively the antibodies to IBR virus could neutralize the isolates (Table II).

**Table I**

Isolation of IBR virus from test material

| | Nasal discharge | Conjunctival discharge | Stool | Lung | Nasal mucosa | Tonsil | Spleen + liver |
|---|---|---|---|---|---|---|---|
| Calves in acute stage | 18/4* | 15/1 | 4/0 | 3/1 | 3/0 | 3/2 | 3/0 |
| Calves with pneumonia | 15/0 | 15/0 | 0 | 8/0 | 8/0 | 5/0 | 3/0 |
| Growing cattle in acute stage | 6/1 | 5/0 | 0 | 1/0 | 1/0 | 1/0 | 1/0 |
| Cows in acute stage | 10/0 | 5/0 | 0 | 2/0 | 1/0 | 0 | 2/0 |

* The numerator shows the number of test materials, the denominator that of positive virus isolations

**Table II**

Identification of three isolates by virus neutralization test

| Virus strain | Antiserum titres | | | |
|---|---|---|---|---|
| | IBR virus | Aujeszky's disease virus | Bovine adenovirus | Rhinopneumonitis virus |
| IBR strain Nu/67 | 1 : 64 | 0 | 0 | 0 |
| Isolate BU/68 | 1 : 32 | 0 | 0 | 0 |
| Isolate BO1/68 | 1 : 32 | 0 | 0 | 0 |
| Isolate HO/68 | 1 : 64 | 0 | 0 | 0 |
| Aujeszky's disease virus strain Igar/59 | 0 | 1 : 128 | 0 | 0 |
| Bovine adenovirus strain THT/62 | 0 | 0 | 1 : 128 | 0 |

Examination of paired sera of cattle from the infected herds revealed the presence of virus-neutralizing antibodies to IBR virus in the second blood sample. While such antibodies were invariably present in second blood samples from cows and fattener bulls, they were absent from about one third of convalescent calf sera. The calves had considerably lower antibody titres (1 : 2—1 : 16) than the adult cattle (1 : 4—1 : 256). Antibodies were also found in the sera of some symptomless animals. The paired sera were also examined for the presence of antibodies to bovine virus diarrhoea (BVD) virus and adenoviruses. If such antibodies were found at all, titre rises did not occur.

From the lungs of spontaneously died, or emergency-slaughtered animals, corynebacteria and streptococci were isolated.

## Discussion

In Hungary, the mass incidence of a febrile respiratory disease with non-characteristic symptoms, but obviously of an infectious nature, has recently been observed in numerous large cattle herds. The disease chiefly affected the

1—6 months old rearing calves and seldom occurred among growing cattle or dairy cows. About 50—80% of the calf herd developed acute symptoms (41—42 °C body temperature, listlessness, serous nasal discharge, foamy salivation, reddening of the muzzle, difficult breathing) which progressed to pneumonia as complication in the majority of the cases. A fatal outcome was quite rare during the acute stage, but more than 20% of the calves that had developed catarrhal-purulent pneumonia succumbed or had to be emergency slaughtered. The disease took a benign course above 6 months of age: the severe acute symptoms rarely progressed to pneumonia, thus losses did not exceed 1—2%. It is remarkable that neither calves nor adult cattle showed the ulcerations characteristic of infectious bovine rhinotracheitis in the upper airways (nasal cavity, trachea).

It is known from the literature and our own experience that among the diseases caused by IBR virus the infectious rhinotracheitis of fattener cattle and cows is manifested in the form of a characteristic ulcerative condition of the upper respiratory tract. Mass incidence of a general respiratory disease due to IBR virus was not previously reported among rearing calves. General respiratory symptoms without characteristic change of the upper airways were observed by BAKER *et al.* (1960) in experimentally infected young calves and by KRUININGEN and WOLKE (1963) in a few individuals of a large calf herd, but these data did not at first lead us to the suspicion that the aetiological agent of the outbreaks might be the IBR virus.

Initially the condition was attributed to respiratory infection by bovine adenoviruses. However, we failed to isolate adenovirus, and adenovirus antibodies, when found at all, had equal titres in the early and convalescent serum samples. Bovine viral diarrhoea could be excluded on a similar basis.

IBR virus could be isolated in cell culture from several diseased calves, but the attempts to isolate virus from the majority of acutely diseased calves and from cows failed. We cannot offer any explanation for the negative results. It was also remarkable that one third of the convalescent calves had no measurable antibody.

This general respiratory form of IBR can scarcely be differentiated from other viral respiratory diseases on the basis of symptoms. Precise identification requires the demonstration of a rise in the specific neutralizing antibodies. The modes of diagnostic differentiation will be reported elsewhere.

## Summary

A febrile disease with general respiratory symptoms has recently caused severe losses in herds of 1—6 months old rearing calves and less often among growing cattle and breeder cows. Fever up to 41—42 °C, listlessness, inappetence, serous nasal discharge and foamy salivation were the most common symptoms. Breathing was difficult and, infrequently, there was a painful, dry cough. Part of the animals recovered from the acute illness, the remainder developed a chronic catarrhal-purulent pneumonia. The losses exceeded 20%. In adult animals

the acute, non-characteristic, respiratory symptoms took a benign course and did not progress to pneumonia. From the diseased calves IBR virus could be isolated in tissue culture.

The disease is epizootologically and clinically distinct from the five known pictures (upper respiratory disease, viral vaginitis, encephalitis, conjunctivitis, abortion) caused by IBR virus, and can therefore be regarded as the sixth disease entity due to this agent.

In Hungary, the sixth IBR virus disease has caused more losses in itself than the other pictures together.

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Address of the authors: Dr. Adorján BARTHA, Dr. János KISARY, Dr. Sándor BELÁK. 1581 Budapest, Pf. 22, Hungary.

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# ELECTRON MICROSCOPE STUDY OF CHICKEN PROVENTRICULUS

By

Irén HORVÁTH

Department of Anatomy and Histology (Head: GY. FEHÉR),
University of Veterinary Science, Budapest

(Received March 27, 1973)

The thick wall of the avian proventriculus (pars glandularis ventriculi) consists, like the other parts of the alimentary tract, of three layers (tunica mucosa, tunica muscularis and tunica serosa).

The specific functional part is the mucosa, above all the mucous epithelium. Epithelial tissue dipping into the underlying propria forms the crypt-like necks of the so-called glandulae proprial or "proprial glands" and the glandular lobules in the submucosa. The latter have been termed "submucosal glands", on the basis of their localization.

The ventricular lumen, the necks of glandulae propriae, the alveoli of submucosal glands and the epithelial lining of tubular outlets can be differentiated from one another by light microscopy. Electron-microscope studies have as yet only been reported on submucosal glands (SELANDER, 1963; TONER, 1963, 1971). The electron-microscopic examinations reported in this paper cover all epithelial cell types of the mucous membrane.

The proventriculus of the avian embryo begins to separate from the gizzard on the fifth day of incubation (DAWSON and MOYER, 1948; cit. BELL and FREEMAN, 1971). According to KUO and SHEN (1936; cit. BELL and FREEMAN, 1971), at 8—9 days there are already indications of peristalsis. LUPPA (1962) and TONER (1965) reported that secretion of gastric acid and enzymes set in at 11—13 and 12—16 days, respectively, in connection with swallowing of protein by the embryo. From the 18th or 19th day of incubation, the embryonic proventricular glands were found to be morphologically identical with those of the adult bird (LUPPA, 1962).

The shape, structure and localization of submucosal glands were investigated by SCHREINER (1900; cit. KOVÁCS 1928), KOVÁCS (1928), CALHOUN (1961), GUZSAL (1963) and MICHEL (1971) and histochemical studies were reported by CHODNIK (1947; cit. BELL and FREEMAN, 1971), LUPPA (1962), MICHEL (1971), and BELL and FREEMAN (1971). BROWNE proposed already in 1922 that the avian proventriculus is analogous to the mammalian stomach, and TONER (1963) and SELANDER (1963) supported this by studying the ultrastructure of the so-called oxynticopeptic cells of birds, cells producing both gastric acid and enzymes.

## Materials and Methods

Healthy, adult White Leghorn and Lohman hybrid chickens (75% roosters, 25% layers) were used. Specimens for electron-microscopy were fixed in a 2.5% glutaraldehyde solution and, subsequently, in Millonig's 1% $OsO_4$ solution for 90 minutes. After dehydration in step-graded ethanol, the specimens were embedded in Durcupan ACM and cut into sections in a Reichert

$OmU_2$ ultra-microtome. Semi-thin sections were stained with 1% toluidine blue, ultra-thin sections were counterstained with uranyl acetate and lead citrate. The electron micrographs were prepared in the Department of Pathological Anatomy, University of Veterinary Science, Budapest, using a TESLA BS 613 type electron microscope.

## Results

The tall columnar cells of the mucous epithelium bear relatively few, short microvilli (Fig. 5). The cytoplasmic processes of the latter are covered by the cell membrane, associating at the surface with a delicate filamentous layer, the mucopolysaccharide coat, corresponding to one form of the glycocalyx. The lateral cell membranes have apically smooth contours, but extend many cytoplasmic processes at the basal part, thus increasing the cell surface (Fig. 6). The neighbouring cells are linked apically with a terminal bar, followed by desmosomes, common membraneous appositions and wide intercellular spaces (Figs 7—8). Invaginations of the cell membrane at the basal part partition the cytoplasm in that region. There is distinctive basement membrane beneath the epithelial cells. Mucin granules of irregular shape and variable electron density localize in the supranuclear region of the electron-dense cytoplasm. Each granule is surrounded by a limiting membrane. Some of the granules are associated with the cell membrane, others are patent towards the luminal side, having obviously emptied the contents into the lumen (Figs 7—8). The mitochondria have diverse shapes and the bulk of them localizes in the supranuclear zone. The well-developed Golgi-apparatus is localized supra- and paranuclearly. Apart from free ribosomes, a distinctive coarse endoplasmic reticulum is seen in the perinuclear zone of the cytoplasm. The elongated nucleus is basally placed and contains a medium amount of chromatin. The chromatin pattern varies with the fixation technique, but chromatin usually aggregates nearby the nuclear membrane. There is a prominent nucleolus (Fig. 6).

The cells of the glandulae propriae are somewhat less tall than the superficial mucin-producing cells, but do not notably differ in structure from the latter.

The lining epithelium of the submucosal gland outlet consists of tall columnar cells (Fig. 2). The apical parts of the cells are slightly bulging into the tubular lumen and only very few cytoplasmic processes extend from the cell surface. The cells are linked basally by a terminal bar, laterally by intricate interdigitations; in places intercellular space and desmosomes are seen between neighbouring cells (Fig. 9). The cytoplasmic membrane forms creases and folds at the cell base. A distinctive basement membrane separates the epithelium from the connective tissue (Fig. 10). Collapsed tubules and vesicles

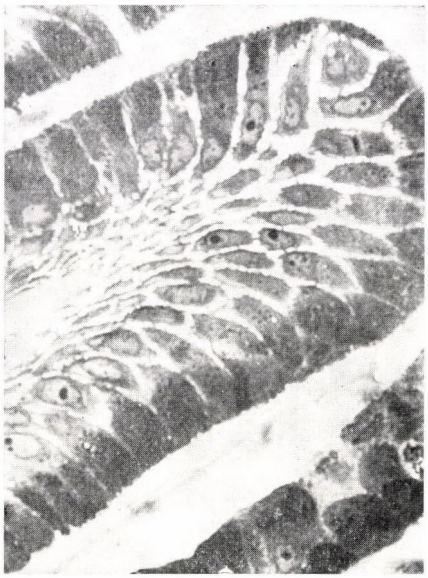


*Fig. 1.* Proventricular lining and crypt epithelium. Semi-thin section, toluidine blue, ×1000

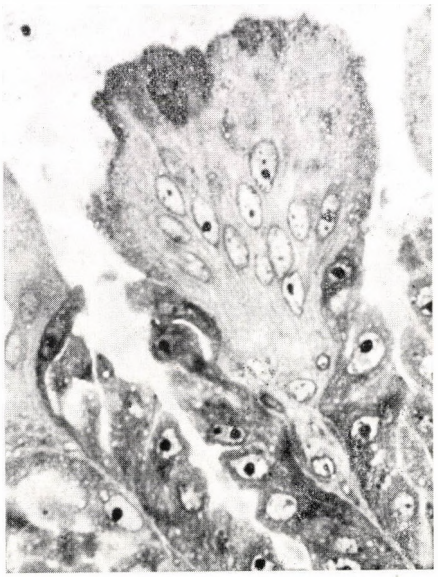


*Fig. 2.* Transition between submucosal gland and duct epithelium. Note mucin in the apical part of one cell. Semi-thin section, toluidine blue ×1000

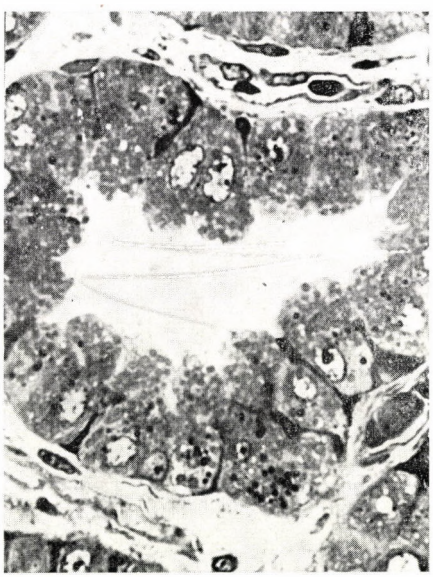


*Fig. 3.* Tubular neck of submucosal gland in transversal section. Note details of undifferentiated cells among the oxyntico-peptic cells. Semi-thin section, toluidine blue, ×1000

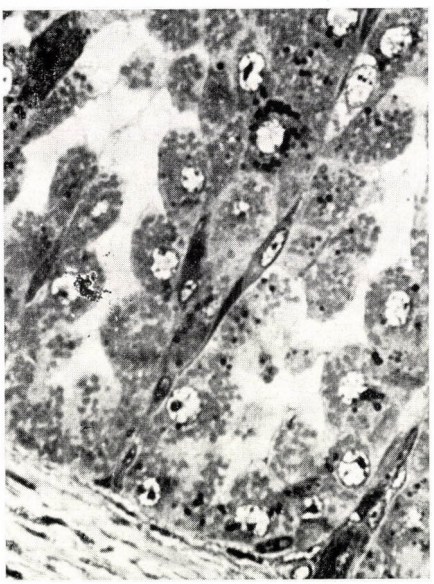
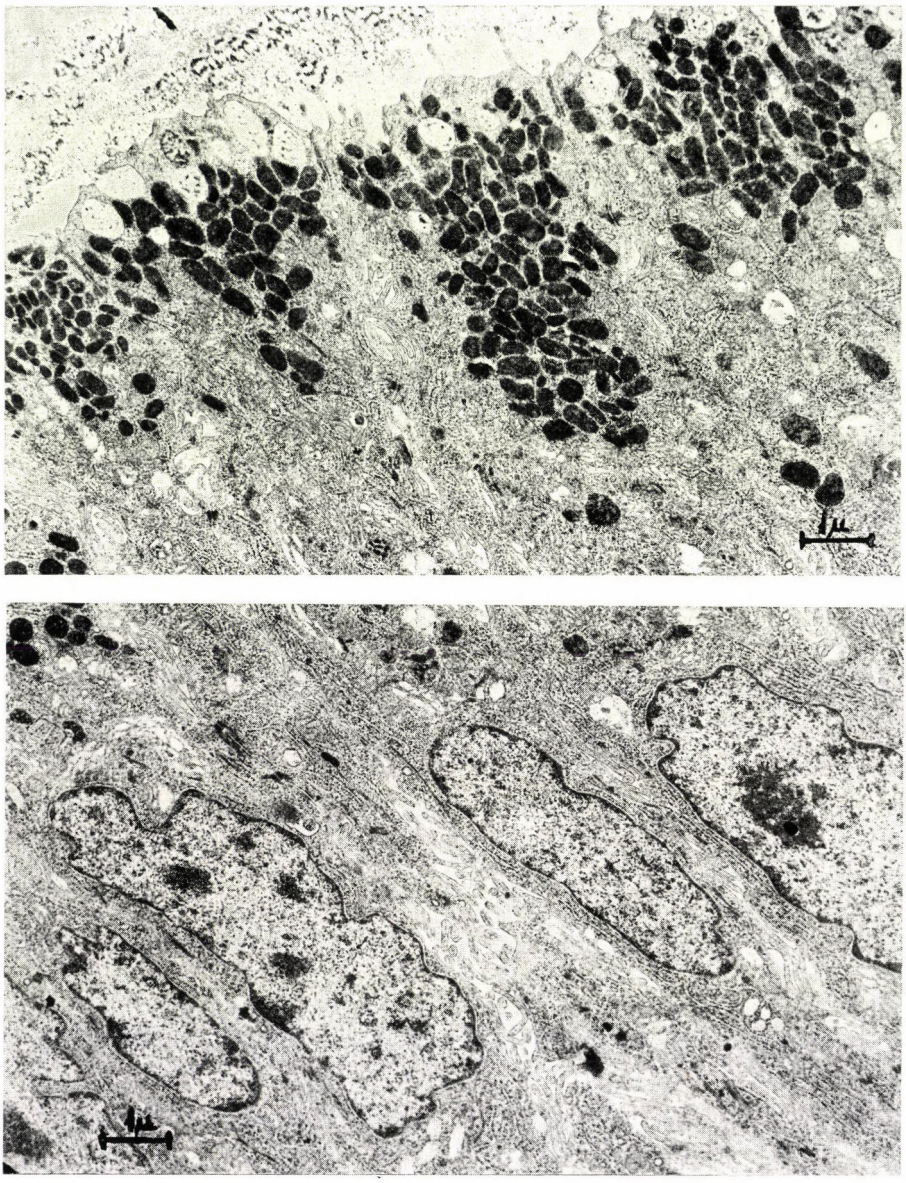
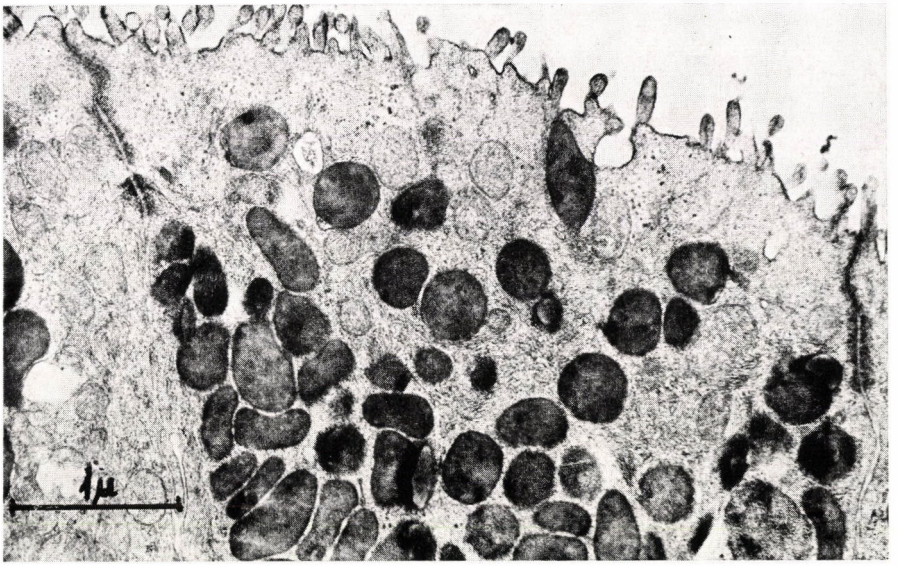


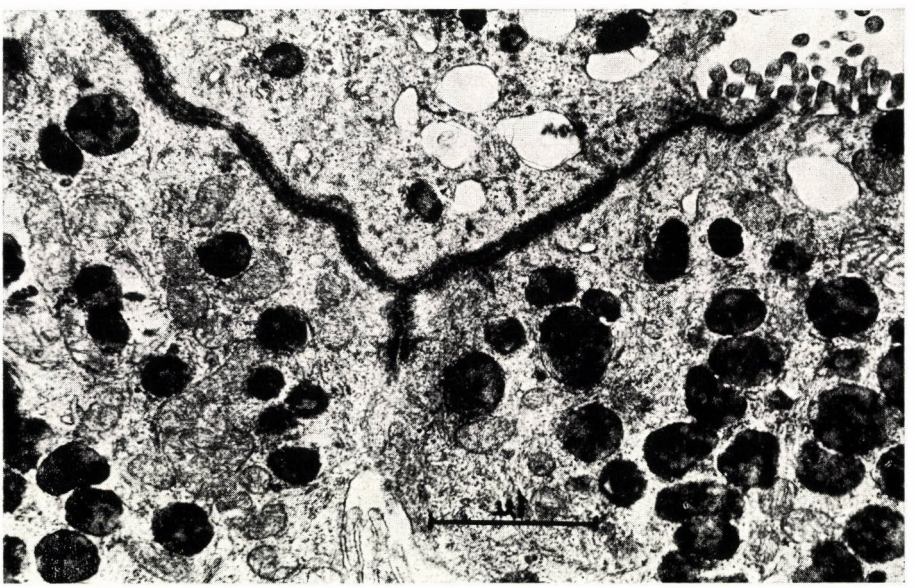
*Fig. 4.* The apical part of oxyntico-peptic cells dips into the tubular lumen. In the cytoplasm, mitochondria and granules of secretion. Semi-thin section, toluidine blue ×1000



*Figs 5—6.* Lining epithelium of proventricular lumen and crypts. Mucin granules localize in the apical region; nearby the surface some granules are empty. The intercellular spaces are more distinct basally than above. ×9723

are seen in the apical part of the cytoplasm. The relatively short mitochondria characteristically localize above the nucleus; these contain few cristae and vary in shape, being round, elongated or irregular (Fig. 9). The indistinct endoplasmic reticulum localizes in the perikaryon. The cytoplasm is inter-



*Figs 7—8.* Mucin granules of various shape, size and electron density localize in the apical parts of luminal and crypt epithelium cells. Some granules are empty, with only the limiting membrane left. A terminal bar links the apical parts of the cells with one another. ×21,000

wined by a delicate filamentous network. Most ribosomes are free, but some are associated with the endoplasmic reticulum.

The cuboidal or low columnar oxyntico-peptic cells are the characteristic component of the submucosal glands (Figs 3—4). These cells, performing the

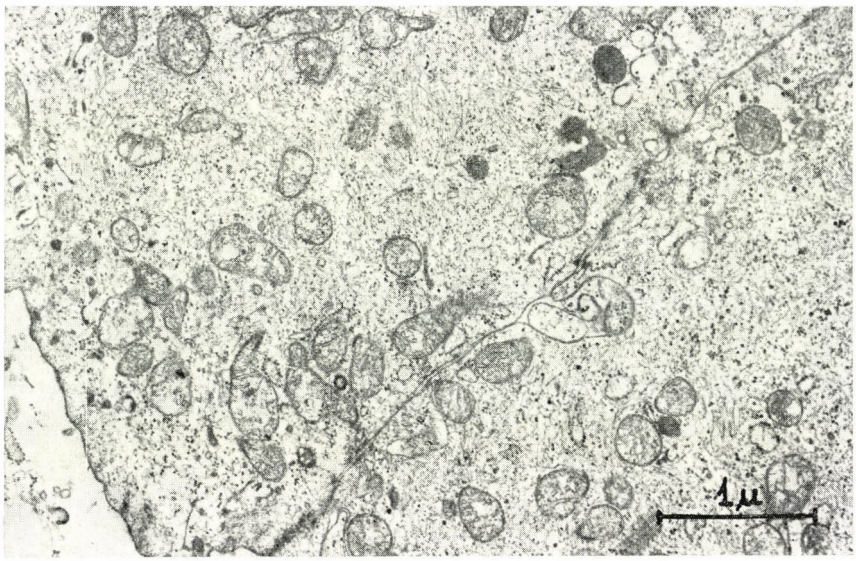



*Fig. 9.* Apex of epithelial cell from glandular outlet, containing round and irregular mitochondria and a delicate mesh of filaments (cytofilaments). ×19,800

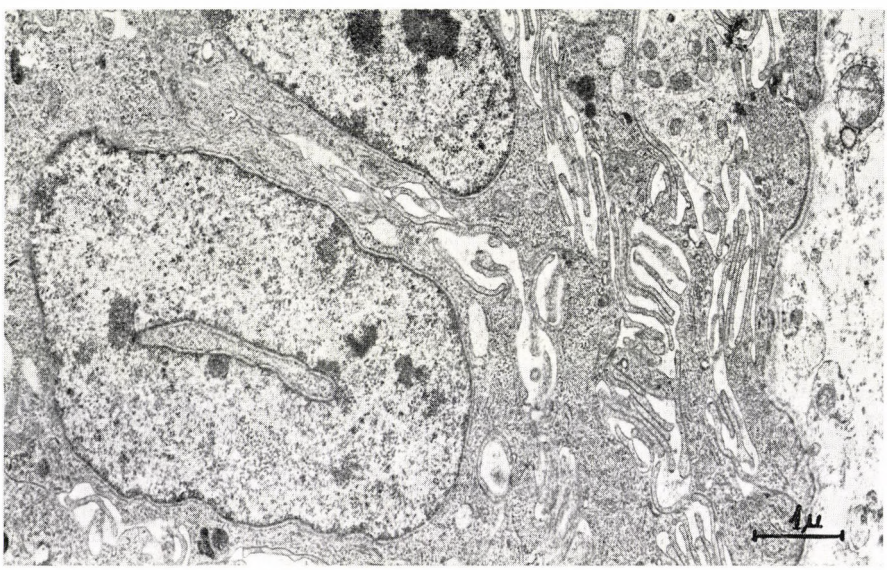



*Fig. 10.* Perinuclear and basal regions of the cell shown in Fig. 9. A cytoplasmic invagination extends into the nucleus. Note partitions at the basal part. ×10,649

functions of both of mammalian chief cells and oxyntic cells, possess the combined properties of both. Changes of shape and structure are closely related with the given functional state of the cell. If the stomach is empty, the submucosal gland epithelium cells widen at the basal part, the narrowed apex

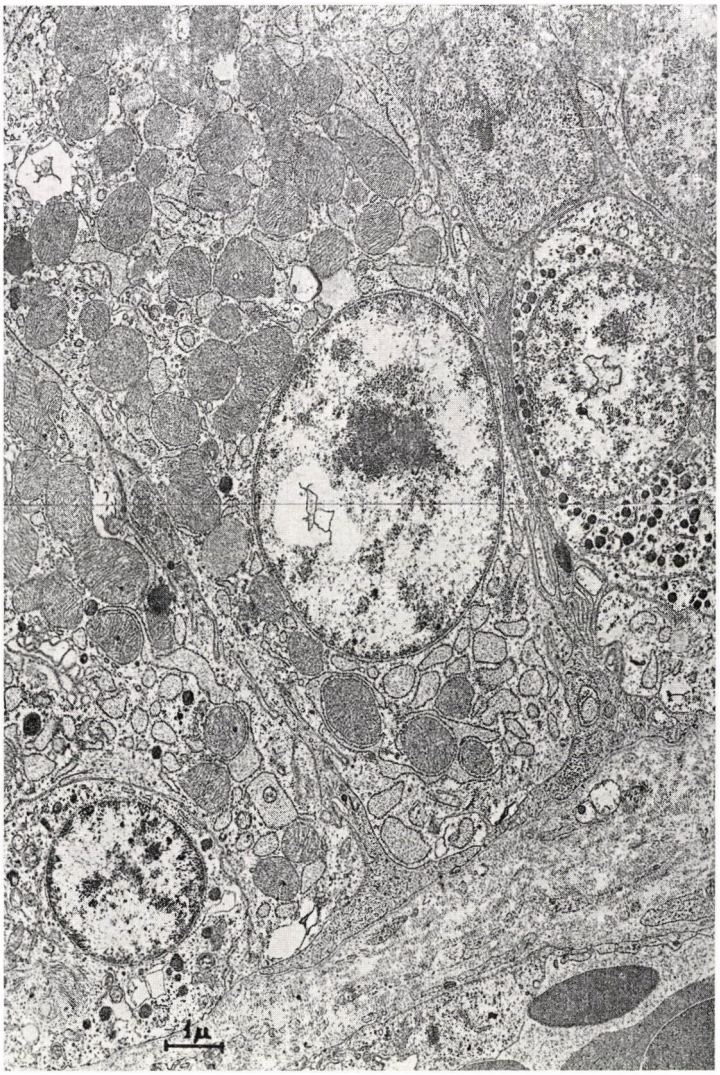



*Fig. 11.* Argentophilic cells wedged between oxyntico-peptic cells. Detail of a capillary vessel is seen in the loose fibrous tissue beneath the basement membrane. ×6945

bulges into the tubular lumen and the free surface of the cell is mooth. The neighbouring cells contact apically only by loose cytoplasmic processes and there is a shift of the terminal bar towards the basal cell region so that a closer contact exists only between the basal two-thirds of the cells. Intercellular junction is represented by interdigitation. The basal cell surfaces are increased by invaginations of the cytoplasmic membrane at the basal part. The main characteristic of such cells is the presence of many large mitochondria; (Figs

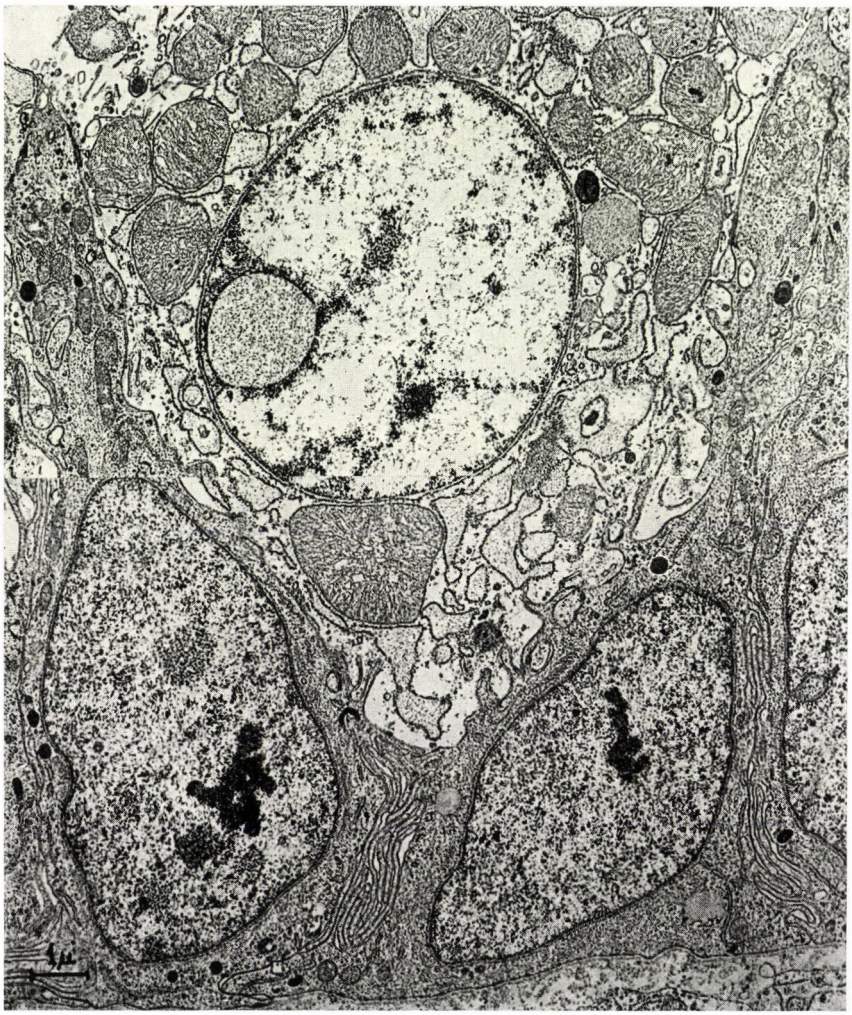
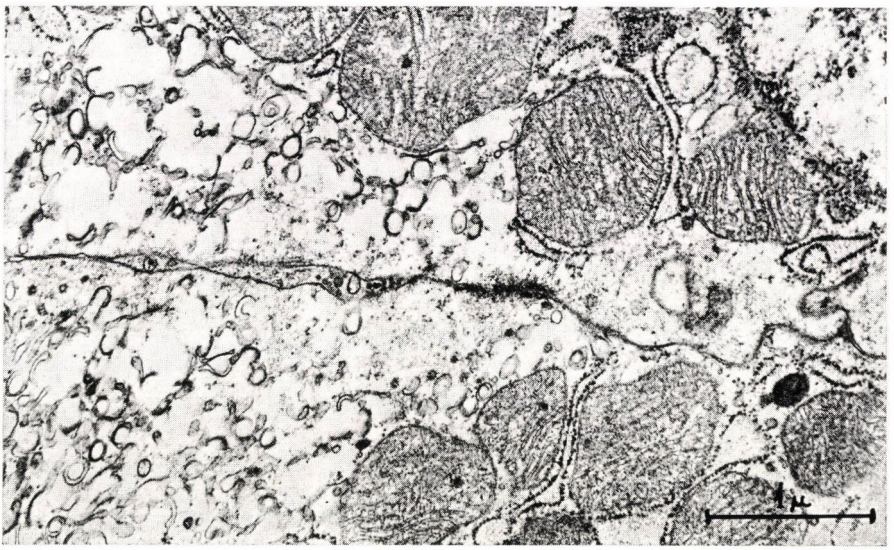
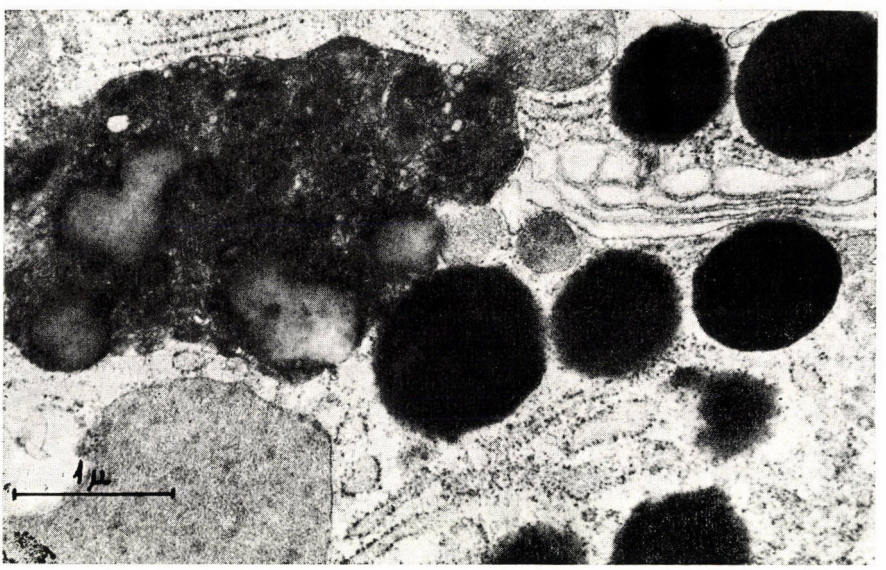


*Fig. 12.* Oxyntico-peptic cell with an intranuclear inclusion, surrounded semicircularly by undifferentiated cells. The endoplasmic reticulum is markedly distended. ×6945

11—12) these are round, with many cristae, and localize supra- and paranuclearly. A system of vesicles and tubules is found in the apical part of the cells (Fig. 13). The well-developed coarse endoplasmic reticulum localizes perinuclearly and basally (Figs 11—12). A rich Golgi complex extends around the nucleus (Fig. 14). Granules of secretion are chiefly found in the apical region, but several paranuclear granules are also seen. The zymogen granules are homogeneous and vary in electron density (Figs 4, 16). Lysosomes vary in number and size (Fig. 14). The nucleus is large, round, rich in chromatin and is slightly basally placed. Chromatin is abundantly present in the karyoplasm






*Figs 13—14.* Cytoplasmic organelles of oxyntico-peptic cells. There are many mitochondria, tubules and vesicles, a bulky, distended coarse endoplasmic reticulum, a well-developed Golgi complex, lysosomes and granules of secretion. ×21 000

and the nucleolus is prominent (Figs 4, 11). Nuclear inclusions, surrounded by a limiting membrane and differing in density from the nucleus, are occasionally also seen (Figs 12, 15). Empty cells are slimmer and taller than those carrying secretion, their free surface is smoother and intercellular contact is

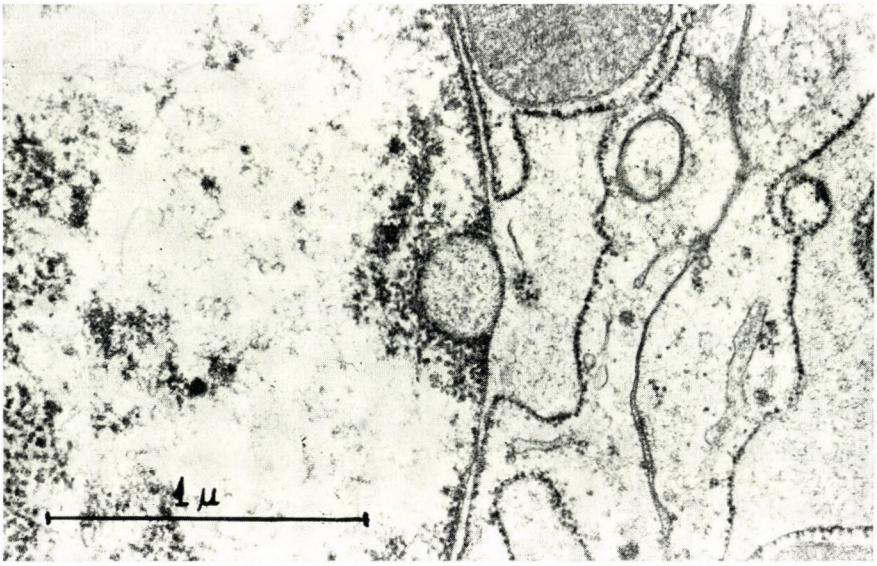



*Fig. 15.* The close joint of the nuclear membrane and endoplasmic reticulum is well shown. In the caryoplasm there is a globular electron-dense inclusion. ×42,000

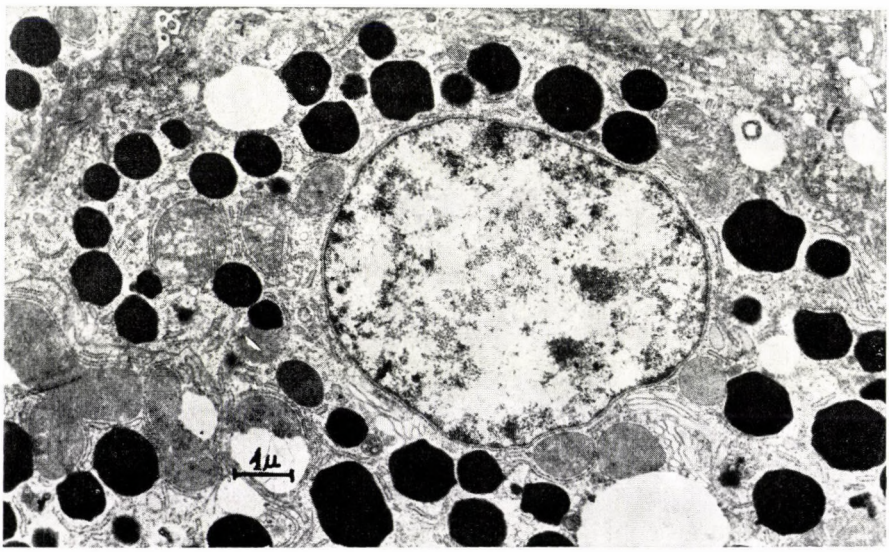



*Fig. 16.* Resting oxyntico-peptic cell. Many zymogen granules accumulate in the cytoplasm in this phase. ×6945

closer even above the terminal bar (Fig. 11). Cells beneath the terminal bar develop more complex interdigitations. In resting cells the number of vesicles is markedly decreased and the secretion granules are empty. The granular endoplasmic reticulum dips deeper into the apical cytoplasm, and its distended

ductuli are filled by an electron-dense, homogeneous substance (Figs 11, 12, 15). The nucleus becomes slightly displaced towards the luminal surface.

Argentaffin cells also occur among the mature and undifferentiated ("fibrillovesicular") oxyntico-peptic cells (Figs 11, 12). These localize along the basement membrane between columnar epithelium cells. The cytoplasm contains uniform, round, membrane-coated electron-dense granules, an undeveloped endoplasmic reticulum and a few, small mitochondria. The nucleus is round and centrally placed.

## Discussion

It is generally accepted that the gastric juice of birds is produced by cells localizing in the thick wall of the proventriculus.

The majority of investigators KOVÁCS, 1928; LUPPA, 1962; GUZSAL, 1963; MICHEL, 1971) are in accord that the gland epithelium cells localize in the propria (or superficial propria) and submucosa (or deep propria), but fail to agree in respect of the presence or absence of a muscularis mucosae.

Others (e. g. CALHOUN, 1961) have doubted the existence of superficial glandulae propriae, stating that mucosal folds and recesses are coated by a simple columnar epithelium.

In the present study no notable ultrastructural difference was observed between lining epithelial cells of the proventricular lumen and glandulae propria cells. The columnar epithelial cells resemble in structure the mucin-secreting cells lining the lumen and recesses of the mammalian stomach. Accordingly, the submucosal glands of the avian proventriculus are analogous to the grooves (sulci) of the mammalian stomach.

The avian proventriculus was first compared with the genuine mammalian stomach by BROWNE (1922; cit. KOVÁCS, 1928). Subsequent light-microscopic studies disclosed the intricate system of submucosal glands and the exocrine nature of the gland epithelium (AITKEN, 1958; LUPPA, 1962; CALHOUN, 1961; GUZSAL, 1963). LUPPA (1962) concluded from topochemical studies that production of HCl and pepsin is also taking place in the proventriculus, but this awaited later electron-microscopic evidence. The ultrastructure of submucosal gland epithelium cells was found to be an intermediary type between the mammalian chief cells and oxyntic cells (SELANDER, 1963; TONER, 1963) and the term "oxyntico-peptic cells" was coined on a functional basis. The number of intracytoplasmic zymogen granules, vacuoles and vesicles, as well as the cell shape differed in fasting and histamine-stimulated birds (SELANDER, 1963; TONER, 1963). According to CHODNIK (1947), each cell is an independent functional unit, whence they do not release secretion simultaneously. In the present study, all oxyntico-peptic cells were found to be in the same stage of secretory activity.

Under physiological circumstances poultry consume food *ad libitum;* thus, taking into consideration the storage of food in the crop, gastric secretion may well take place continuously. Apart from the quality and quantity of food, the part of the day also plays a role in the intensity of secretion. As no food is consumed overnight, zymogenic granules and vacuoles accumulate in the cells — probably under neurohormonal regulation — and their quantitative increase alters the morphology of the cell. Food intake in the morning is followed by release of the secretion accumulated overnight. The morphological signs of this process are, among others, the release of zymogen granules, decrease of intra-cytoplasmic vacuoles in number, increase of the cell surface, *etc.* This can account for the finding that the oxyntico-peptic cells of chickens killed at dawn resemble those of starved chickens whereas feeding or stimulation with histamine gives rise to similar changes in these cells.

## Acknowledgements

The author is indebted to Dr. E. GUZSAL, Associate Professor, for valuable advice, to Dr. P. KAPP, Associate Professor and his coworkers for preparing the electron micrographs. Thanks are due to Dr. B. LOVAS, Senior Research Worker, for the instructions kindly given to the author in electron microscopic techniques.

## SUMMARY

Cell types of the mucous epithelium of avian proventriculus were studied by electron microscopy in specimens from chickens of both sexes.

Cells of the lining epithelium of proventricular lumen and the epithelial cells of "glandulae propria" were found to be similar in structure to the superficial mucin-secreting cells of the mammalian stomach. As the lining epithelial cells and crypt cells did not notably differ in any ultrastructural detail, the terminological distinction of the latter as "glandulae propriae or proprial glands" seems to be unnecessary.

The greater part of the proventricular wall is occupied by the submucosal glands, which form many roundish or irregularly-shaped lobules. The tubular alveoli are, like in the lower vertebrates, lined by the so-called oxyntico-peptic cells. The ultrastructure of these cells owing to their dual function represents a special intermediary type between the chief cells and oxyntic cells of mammals. The oxyntico-peptic cells perform synchronously a continuous secretory activity. As poultry consume food *ad libitum,* the intensity of secretion chiefly depends on the part of the day.

Among mature oxyntico-peptic cells, undifferentiated forms and "argentophilic" cells were also encountered.

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The address of the authoress: Dr. Irén HORVÁTH, 1078 Budapest, Landler J. u. 2, Hungary.

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# DATA ON THE "OCTOMITOSIS" (SPIRONUCLEOSIS) OF CYPRINIDS AND AQUARY FISHES

By

K. MOLNÁR

Veterinary Medical Research Institute (Director: D. DERZSY),
Hungarian Academy of Sciences, Budapest

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The occurrence of diplomastigine flagellates in fish intestine has long been known but only the species parasitic in salmonids have been studied in greater detail (SCHMIDT, 1920; MOORE, 1923; DAVIS, 1926). Scanty data are available on the diplomastigines of aquary fishes (SCHÄPERCLAUS, 1954; REICHENBACH—KLINKE, 1966; KULDA and LOM, 1964a), and only brief reference has been made to such parasites of cyprinids (SCHMIDT, 1920; REICHENBACH—KLINKE, 1966; CHEN, 1956).

A disease of the fry, usually referred to as "octomitosis", is commonly occurring in trout pond farms. KULDA and LOM (1964b) have attributed it to *Hexamita salmonis* (MOORE, 1923), a species mentioned by SCHÄPERCLAUS (1954) under the name *Octomitus truttae*. According to SCHÄPERCLAUS, this parasite occurs not only in trouts, but also in aquary fishes (*Pterophyllum*, *Cichlasoma* and *Heterandria* species), but KULDA and LOM (1964a, b) showed that *Hexamita salmonis* is strictly specific for salmonids and as such distinct from the parasite found in the angel fish *(Pterophyllum scalare)*, which they identified as the amphibian parasite *Spironucleus elegans* (Lavier, 1936). REICHENBACH—KLINKE (1966) agreed with KULDA and LOM, but remarked that hexamita-like parasites probably belonging to the species *Spironucleus elegans* (Lavier, 1936) also occur in the common carp. This accords well with the earlier observation of SCHMIDT (1920), but attempts for the experimental transfer of *Spironucleus elegans* to cyprinids indigenous in Europe failed (KULDA and LOM, 1964b). Out of the cyprinids other than the common carp, only the grasscarp *(Ctenopharyngodon idella)* was found to harbour a diplomastigine species in the gut, which had originally been described in China (CHEN, 1956) as *Hexamitus* sp. but, on the basis of the original description, KULDA and LOM (1964a, b) have believed it to belong to the genus *Spironucleus*.

The pathogenic effect of hexamites depends, according to DAVIS (1956), chiefly on environmental factors and death is caused by enteritis. SCHÄPERCLAUS (1954) and REICHENBACH—KLINKE (1966) reported that *Hexamita (Octomitus) salmonis* was occasionally found in the blood of trouts and *Hexamita (Octomitus) intestinalis* in that of frogs and tortoises.

No literary data are available on the pathogenic importance of diplomastigine flagellates parasitic in cyprinids and little is known about the similar parasites of aquarium fishes. The present study contributes some new data to the knowledge of diplomastigins and of the pathomechanism of the disease caused by them, on the basis of fatal infections occurring in certain fish species indigenous in Hungary.

## Material and Methods

The examined fishes originated from various pond farms, from the river Danube and from aquarium breeds.

In the majority of the cases, only the occurrence of parasites was established, but if the host showed symptoms, detailed examinations were car-

ried out. In such cases the parasites found in the fresh carcases or in killed fishes were examined in stained and unstained preparations. The smears were stained with the techniques of Giemsa, Pappenheim and Farkas-Mallory after fixation in methanol. The affected organs were fixed in 4% formalin, embedded in paraffin, and the sections were stained with haematoxylin and eosin or with the Farkas-Mallory technique.

## Results

Diplomastigine flagellates were often found in the gut of cyprinids. The majority of grasscarps *(Ctenopharyngodon idella)* harboured the *Spironucleus (Hexamitus)* sp. described by CHEN (1956).

Similar parasites were occasionally encountered in the gut of bigheads *(Hypophthalmichthys nobilis)* and common carps *(Cyprinus carpio)* from pond farms and in that of *Chondrostoma nasus* and *Barbus barbus* fishes taken from the river Danube. Aquarium fishes, above all the angel fish *(Pterophyllum scalare)*, were also found to harbour diplomastigines.

### *Morphological observations*

All diplomastigines were characteristically equipped with 3 pairs of anterior flagellae and one pair of posteriorly pointed flagellae. These structures were only seen in unstained preparations, in the premortal stage when the movements of the parasite had slowed down. In stained preparations (Giemsa, Pappenheim) there was no indication of flagellae, only of their intracellular portions, which took on a pale blue stain against the darker blue cytoplasm and the red-staining nucleus. In smears stained with Farkas-Mallory's technique, many vacuoles became apparent in the homogeneous pink cytoplasm, which, like the nucleus, did not take on the stain. The protozoon can be recognized by the poor staining affinity of the nucleus and vacuoles also in histological sections of necrotic liver tissue (Fig. 2). The measurements of the parasites are almost identical in live and fixed state; diplomastigines from the liver of *Barbus barbus* measured 10—12 by 5—9 $\mu$m, those from angel fish 7—9 by 3—5 $\mu$m and those from grasscarp 8—10 by 4—7 $\mu$m. All parasites had an oval or long oval shape.

The generic classification of diplomastigine flagellates *(Octomitus, Hexamita, Spironucleus)* is based on nuclear morphology. Such studies were not possible in the frame of the present work but, accepting KULDA and LOM's opinion (1964a, b), I have tentatively classified the parasites into the genus *Spironucleus* Lavier. The protozoon found in the grasscarp is clearly identical with CHEN's (1956) diplomastigine, and the identity of the aquary fish parasite with *Spironucleus elegans* Lavier, 1936, is at least very probable. Since,

however, literary data on the diplomastigine of *Barbus barbus* are lacking, it has remained to be clarified whether the *Spironucleus* parasites occurring in the gut of European cyprinides are identical with *Spironucleus elegans* Lavier 1936 or other known *Hexamita* or *Spironucleus* species, or they represent a new species not previously described. Until information is emerging from detailed studies, I refer to them as *Spironucleus sp.*

### *Pathogenicity of the parasites*

Losses due to *Spironucleus* parasites were observed in three fish species.

*"Spironucleosis" of the grasscarp.* The parasite described by CHEN (1956) as *Hexamitus* sp. and classified by KULDA and LOM (1964a,b) into the genus *Spironucleus* were found in the posterior two thirds of the gut of the grasscarp, occasionally in very large numbers. In some ponds 100% of the grasscarps were infected. The intensity of infestation was greatest among the one-summer and two-summer fishes. The establishment of many parasites in the gut caused gross lesions: the mucous membrane exhibited reddening over the posterior two thirds of the gut and the vessels of the serous membrane were injected.

Losses attributable to spironucleosis in itself were not observed. A 17 cm long one-summer grasscarp kept in an aquary showed a severe ascites. As the case was suspect of infectious dropsy, sample was taken for bacteriological examination. The volume of the abdomen increased enormously, exophthalmia developed and the scales were slightly ruffled (Fig. 1). On abdominal incision a large amount of yellowish-reddish, jellinaceous serum was discharged from the abdominal cavity. The vessels of the intestinal serous membrane and of the mesentery were injected and the posterior segment of the gut appeared as a red tube among the abdominal organs. Some areas of the liver showed a brownish-grey discoloration. Mucosal scrapings from the purple mucous mem-

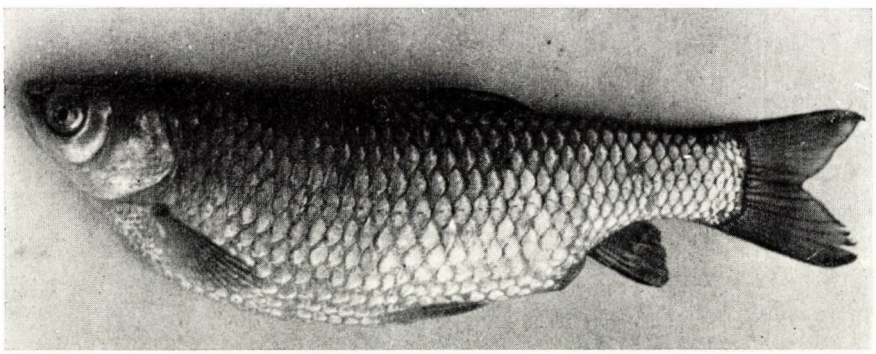

*Fig. 1.* Ascites of grasscarp due to spironucleosis

brane of the posterior gut contained masses of *Spironucleus* parasites. The abdominal exudation was bacteriologically negative, containing only a few red blood cells. Histologically an early stage of necrosis was found in the liver and haemorrhagic enteritis in the gut. The propria of the intestinal mucosal folds and the submucosa contained red blood cells and unidentifiable cellular elements. The vessels were dilated and in places haemorrhages were seen under the serosa. The mucosal epithelium was injured and many necrotic epithelial cells and red blood cells were found in it.

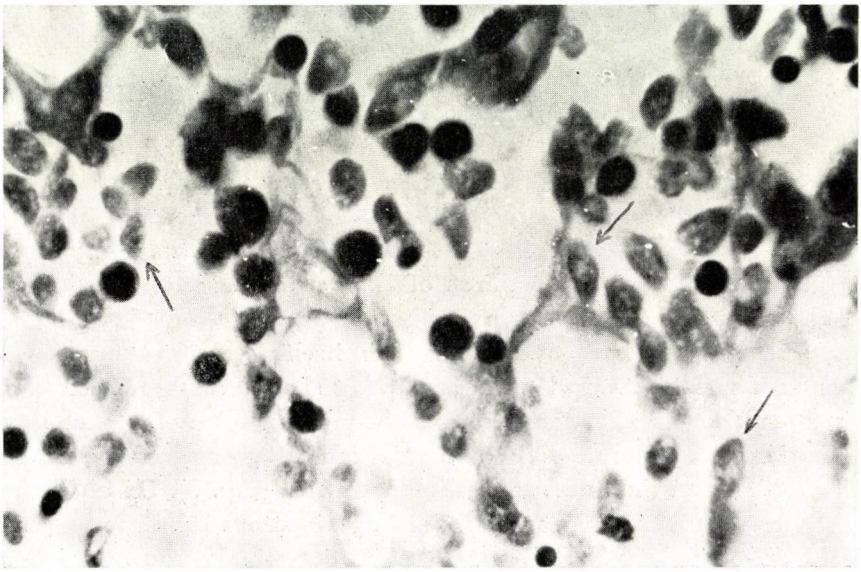

*Fig. 2. Spironucleus sp.* in colliquated liver tissue of a *Barbus barbus* fish

"*Spironucleosis*" *of* Barbus barbus. The fresh carcase of a *Barbus barbus* fish, taken of the Danube river, was markedly cachectic and was therefore examined in greater detail. The backline of the 50 cm long fish was quite sharp and its ribs showed through the skin. On abdominal incision, about 100 ml of yellowish-reddish fluid was discharged from the abdominal cavity. The probosces of *Pomporhynchus laevis (Acantocephala)* were protruding from the serous membrane over an about 10 cm long portion of the gut, spaced at 5 mm distance from one another. The parasites localizing in the lumen were clearly visible through the thinned intestinal wall. In the liver portion localizing in the intestinal loop, at the site where the probosces of *Pomporhynchus* punched the intestinal wall, there was a greenish-yellow, necrotic, friable liver lesion, about 3 cm in diameter (Fig. 3). The necrotic liver portion was packed with *Spironucleus* parasites. About 120 *Pomporhynchus laevis* parasites were found in the exposed intestinal lumen (Fig. 4). Scrap-

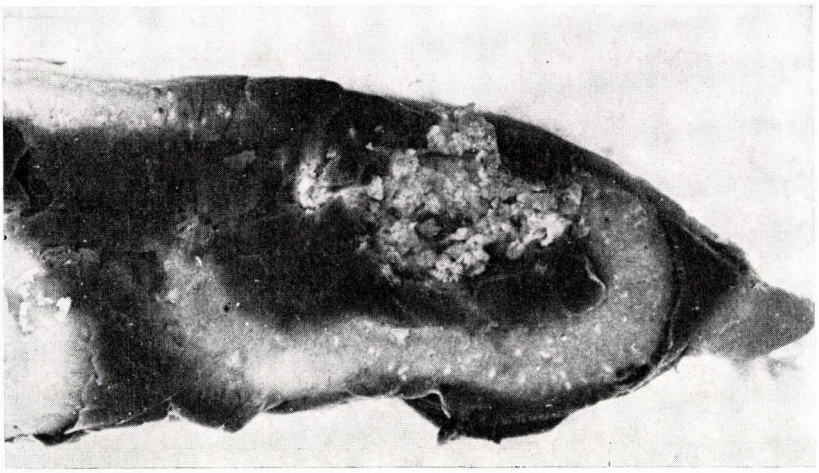

*Fig. 3.* Necrotic detail of liver adjoining the gut portion punched by probosces of *Pomporhynchus* in spironucleosis of a *Barbus barbus* fish



*Fig. 4.* Exposed gut of a *Barbus barbus* fish. Note the many *Pomporhynchus laevis* parasites in the intestinal lumen and residues of necrotic liver portion on the serous membrane

ings from the slightly reddened intestinal mucosa also contained many *Spironucleus* stages, but no parasites were found in the bile and abdominal fluid.

The liver and the gut portion invaded by *Pomporhynchus* and protozoa were examined histologically. The gut showed but little change: apart from cross-sections of *Pomporhynchus* parasites in the lumen, the only ab-

normality was the extravasation of a few red blood cells in the propria. The continuity of mural tissue was interrupted by the probosces protruding into the abdominal cavity and the accumulation of fibrous connective tissue in their surroundings. The probosces and the surrounding connective tissue extended beyond the serous membrane into the liver parenchyma. These parts of the liver were necrotic and the parenchyma transformed to a structureless mass, owing to colliquation necrosis. Apart from colliquated tissues, cell debris and many *Spironucleus* parasites were found in the necrotic liver portions (Fig. 1). In the areas adjoining the intact liver tissue, nuclei deprived of cyto-



*Fig. 5.* Necrotic and relatively sound details of liver parenchyma in a *Barbus barbus*

plasm and groups of disintegrating liver cells were seen. Within intact rows of cells island-like small or larger areas showed early or advanced degeneration (Fig. 5). Swelling and liquefaction of the damaged cells was soon followed by nuclear disintegration.

*"Spironucleosis" of aquary fishes. Spironucleus* parasites were often found in the gut of aquary fishes in the course of routine examinations. In one case losses in an angel fish stock amounted to 50%. Two individuals from this stock, a fresh carcase and an appearently healthy fish were autopsied. The dead fish exhibited reddening of the skin, haemorrhages and ulcerations, especially in the region of the head. *Spironucleus* parasites were abundantly present in muscle specimens taken from the affected parts. The parasites were localized in vessels and in interstitial haemorrhages. Many

*Spironucleus* diplomastigines were also found in the gut, gallbladder and visceral organs. A haemorrhagic enteritis similar to that found in the grasscarp was observed. The healthy appearing fish showed slight catarrhal lesion in the gut and a turbid bile, but no other gross lesions. Masses of *Spironucleus* parasites were nevertheless present in the gut and gallbladder, and a few specimens were even found in impressions of muscle originating from various parts of the body. Both fishes harboured many *Capillaria (Nematoda)* stages in the gut, 32 and 47 specimens, respectively.

## Discussion

As the affected fishes were bacteriologically negative, but masses of *Spironucleus* parasites were found in the changed organs, it seems fairly certain that the losses were due to *Spironucleus* infection. Consideration should be, however, also given to the fact that in every case, predisposing factors had aggravated the course of the parasitic disease.

In the case of grasscarps, long fasting and retention of a small amount of chyme in the gut clearly favoured the multiplication of *Spironucleus* and the development of enteritis. The parasites reached the blood stream and liver across the injured intestinal wall, without infecting the gallbladder. The appearance of exudation in the abdominal cavity was a sequal to haemorrhagic enteritis.

*Spironucleus* sp. can also be held responsible for the liver necrosis developing in *Barbus barbus* fish. The absence of lesions from the gallbladder and biliary ducts suggests that *Spironucleus* stages gained access to the liver through the tissue injuries caused by *Pomporhynchus laevis.* As emaciated *Barbus barbus* specimens are very frequently encountered and mass losses have often been observed among the population in the Danube habitat, it seems highly probable that a complication of *Pomporhynchus* infection by spironucleosis is a much more frequent occurrence that might be inferred from the case described in the foregoing.

The fact that *Spironucleus sp.* was demonstrable in the blood of many aquarium fishes, including those free from symptoms, clearly shows that these parasites, regarded by many authors as harmless gut dwellers, may invade the body of the host and therefore the *Spironucleus* spp. parasitic in the gut of various fish species have to be regarded as organisms capable of causing parasitaemia under given conditions.

It is concluded from the results that the spironucleosis of cyprinids and aquarium fishes is a disease similar in nature to trout octomitosis.

## Summary

Case reports of "spironucleosis" among cyprinids and aquary fishes are presented and the conclusion is drawn that the pathogenic importance of diplomastigine flagellates for these species is similar to that of the so-called octomitosis for trouts.

The diplomastigine flagellates, tentatively classified by the author as *Spironucleus sp.*, occasionally caused losses among grasscarps, *Barbus barbus* fishes and angel fishes. *Barbus barbus* showed enteritis, liver necrosis and serous exudation into the abdominal cavity, whereas angel fishes showed apart from enteritis haemorrhages and ulcerations over the entire body surface, above all in the head region. In dead or diseased fishes, diplomastigine flagellates which are typical gut parasites, also appeared in other organs, in the liver of *Barbus barbus* and in the peripheral blood of the angel fish.

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The address of the author: Dr. Kálmán MOLNÁR, 1143 Budapest, Hungária krt. 21, Hungary.

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# STUDIES ON THE CELL-BOUND *ESCHERICHIA COLI* HAEMOLYSIN

By

F. MURÁNYI and S. JUHÁSZ

Veterinary Medical Research Institute (Director: D. DERZSY),
Hungarian Academy of Sciences, Budapest

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There is little information available on the thermosensitivity of the haemolysin of *Escherichia coli*. LOVELL and REES (1960) described the so-called filterable haemolysin of *E. coli* organisms grown in synthetic media, which was later characterized by SNYDER and KOCH (1966) as thermostable. As in our related studies (MURÁNYI and JUHÁSZ, 1971) the filterable haemolysin effect proved to be due to acids produced by *E. coli* cells its thermostability does not seem to warrant further examinations or explanation.

SNYDER and KOCH (1966) also studied the thermosensitivity of cell-bound *E. coli* haemolysin. *E. coli* organisms grown in Beef Heart Infusion Broth (DIFCO) for 4—10 hours lost haemolysin capacity during a treatment for 1 hour at 56 °C.

The present studies were undertaken to examine the behaviour towards heat of cell-associated *E. coli* haemolysin and to clarify whether there is relationship between the reduction of germ count and fall of the haemolysin titre. The results were evaluated mathematically.

## Materials and Methods

The *E. coli* P 105 (O 138 : K 81) strain, isolated from swine, was used throughout. Treatment at different temperatures was carried out in a waterbath. The haemolysin test was set up and germ counts were determined immediately after heat treatment.

Undiluted culture or its twofold serial dilutions in saline containing 0.005 *M* and a 1% suspension of three times washed sheep erythrocytes were used in the haemolysin test. The results were read after an incubation for 2 hours at 37 °C. Germ count were determined according to MILES and MISRA (1938).

The mathematical evaluation was made by means of a Hewlett-Packard 9810 A computer.

## Results and Discussion

Preliminary experiments showed that while the heat treatment minimized or even stopped the haemolytic activity, it scarcely altered the germ count, if at all. Haemolytic activity ceased at 56 °C already in 30 seconds and at 50 °C

in 15 minutes. Meanwhile the *E. coli* count fell only by about one logarithmic order, which might well have been due to the error of the method.

It follows that the bacterial cell suspension may lose its haemolytic activity without a notable alteration of the count of viable germs. It is, therefore, possible to select a temperature range and time interval within which the reduction of the haemolytic titre can be followed up with the germ count unchanged. Testing of samples every 5 or 10 minutes showed that titre decrease and cessation of the haemolytic activity need a well defineable time of heating. The results are shown in Fig. 1 and Table I.

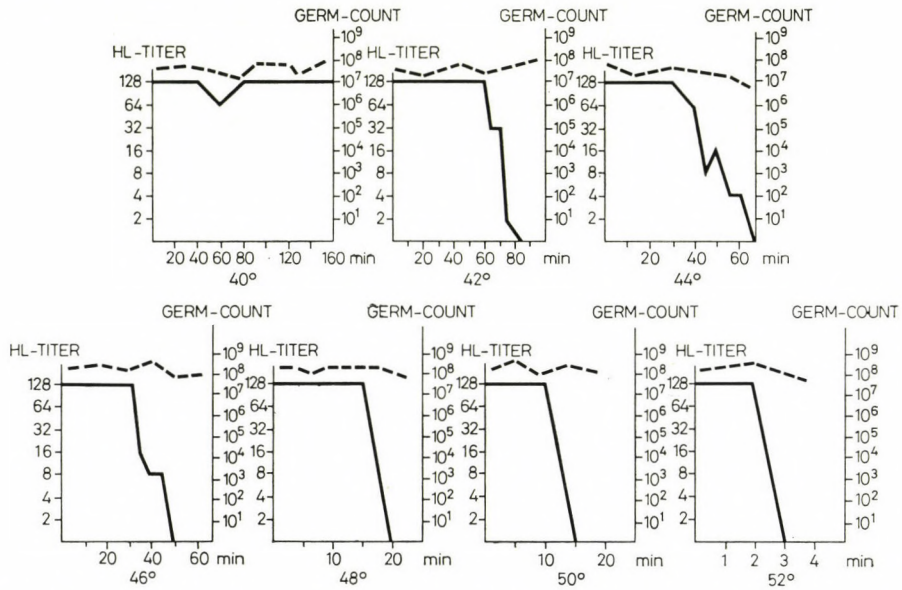


*Fig. 1.* Haemolytic titres and *E. coli* counts during treatments at different temperatures. ——— haemolytic titre; - - - - - - *E. coli* count

**Table I**

Loss of haemolytic activity at different temperatures

| Heat treatment at °C | Beginning of titre decrease (minutes) | Disappearance of activity (minutes) |
|---|---|---|
| 40 | 180 | 180 |
| 42 | 60 | 80 |
| 44 | 30 | 70 |
| 46 | 30 | 50 |
| 48 | 15 | 20 |
| 50 | 10 | 15 |
| 52 | 2 | 3 |

A mathematical correlation was then sought between titre changes and temperature of heat treatment. It was already inferred from the reading of the numerical values that such a correlaton exists in the temperature range between 42 and 50 °C. The mathematical analysis substantiated this implication. Titre changes at 40 and 50 °C differed markedly from the other values measured. Examinations of the values of initial decrease and disappearance of the haemolytic activity in the function of time showed that the phenomenon can best be characterized by an exponential regression. The logarithms of the times of the first appearance of titre decrease were plotted against the temperature of treatment. The results are shown in Fig. 2.

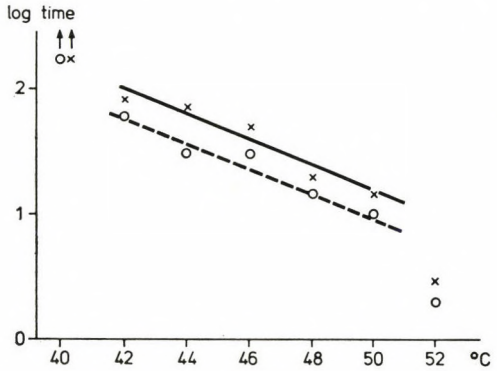


*Fig. 2.* Loss of haemolytic activity in the function of time. 0 and — — — — time of the first demonstrable titre decrease; X and ——— disappearance of activity

The equation of regression for the initial titre decrease is

$$Y' = 5.63 - 0.09\ X,$$

where $Y'$ is the base-10 logarithm of time in minutes and $X$ is the temperature of heat treatment in °C.

At 0.05 probability level the confidence limits are —0.05 and —0.13. The correlation coefficient $r$ differs from 0 at $P < 0.01$ level, indicating a good correlation between the variables $X$ and $Y'$, even with the relatively low number of measurements performed.

The equation of regression for the disappearance of the haemolytic activity is

$$Y' = 6.18 - 0.10\ X,$$

where the dimensions of the values of $X$ and $Y'$ are the same as above and confidence limits at 0.05 level are —0.05 and —0.15.

Similar to the initial titre decrease, the $r$ correlation coefficient (—0.96) differs from 0 at the $P < 0.01$ level.

It seemed important to ascertain whether the two regression lines are in fact parallel. As

$$t = \frac{b_1 - b_2}{S_d},$$

where $b_1$ and $b_2$ indicate the slopes of the regression lines (coefficients of $X$) and $S_d$ is computed by the formula*

$$S_d = \frac{\text{Error } SQ_1 + \text{Error } SQ_2}{SQ_x(n-2)}$$

the result is $t = 0.35$, which corresponds to $P > 0.05$. Thus the slopes of the two lines do not differ significantly, *viz.*, the logarithms of the time of initial titre decrease and of disappearance of the haemolytic activity change parallel in the function of temperature.

As the determinations were carried out at 5-minute intervals and titre measurements were based on twofold serial dilutions, a still greater accuracy of results can be expected from the use of more refined parameters. The curves for changes of the haemolysin titre at 42, 44 and 46 °C on the one hand and at 48 and 50 °C on the other differ in type because more measurements than available would have been required for an accurate plot of the disappearance of haemolysin action. This does not, however, affect the regression correlations demonstrated in the foregoing because these exist between the initial point and end point of the inactivation of haemolysin.

## Summary

Examinations of the cell-bound haemolysin of *E. coli* showed that haemolytic activity disappears on heat treatment, whereas the viable germ count is scarcely affected. In the temperature range from 42 to 50 °C, the time values of the initial titre decrease and of the disappearance of the haemolytic activity showed an exponential regression when plotted out in the function of time. The initial titre decrease and final disappearance of titre ran parallel in the transformed system.

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The address of the authors: Dr. Ferenc Murányi, Dr. Sándor Juhász, 1143 Budapest, Hungária krt. 21, Hungary.

* Description of the calculation of SQ and $SQ_x$ is beyond the scope of this report. Those interested are referred to the book of Sváb (1967).

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# VACCINATION EXPERIMENTS AGAINST TRANSMISSIBLE GASTROENTERITIS (TGE) OF SWINE

## I. RELATIONSHIP OF ANTIBODY LEVEL AND IMMUNITY

By

L, Csontos, T. Szent-Iványi and J. Benyeda

Central Veterinary Institute (Director: A. Ványi) and Department of Epizootiology, University of Veterinary Science (Head: J. Mészáros), Budapest

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The clarification of the aetiology of the transmissible gastroenteritis (TGE) of swine was soon followed by studies on its therapy and prevention.

Swine which recovered from the disease acquire an active immunity, as shown by the appearance of antibodies in blood and colostrum (Haeltermann and Hutchings, 1956; Harada *et al.*, 1967; Gualandi, 1957; Szent-Iványi *et al.*, 1964). The antibodies were demonstrable in animal experiments (Young *et al.*, 1953) and in vitro (Haeltermann and Hutchings, 1956; Lee, 1956; Cartwright *et al.*, 1964; Harada *et al.*, 1967). The colostral antibodies protect piglets from TGE born in an infected environment. If colostral protection is absent, the newborn animals contract the disease and many of them die (Haeltermann and Hutchings, 1956; Szent-Iványi *et al.*, 1964). To ensure colostral protection, investigators in various countries fed to sows exposed to TGE the intestines of piglets died of TGE (Bay *et al.*, 1953; Saunders *et al.*, 1965; Bohl, 1967; Haeltermann, 1963). Such experiments were also carried out in Hungary (Fehérvári, 1969).

Certain authors used virus propagated in tissue culture for this purpose (Thorsen and Djurikovic, 1970), others fed newborn piglets with whole blood or serum from convalescent sows several times on the first days of life (Haeltermann, 1963; Noble, 1964; Saunders, 1964; Vishnyakov and Gorsheva, 1961; Djurickovic and Thorsen, 1970). Attenuation of the virus by tissue culture passages and vaccine production from the attenuated strain was also attempted, using dog kidney (Welter, 1965) or swine kidney cell cultures (Thorsen and Djurikovic, 1970).

Studies on the behaviour in SPF piglets of a TGE virus strain (CKp) adapted to swine thyroid cells have been reported by us (Csontos and Szent-Iványi, 1971).

On the basis of the results obtained on a laboratory scale, an experimental vaccine was prepared from the CKp strain and was tested in a field trial, which is the subject of this report.

### Material and Methods

*Tissue cultures.* Primary pig kidney and secondary swine thyroid cell cultures, prepared as described earlier (Csontos and Szent-Iványi, 1971) were applied.

*Virus strains.* The 42nd tissue culture passage of the CKp strain was used for vaccine production. The centrifuged supernatants of 1 : 10 organ suspensions prepared from intestines of piglets died of TGE were used for chal-

lenge. The infectious material was prepared before the experiments and was stored at —20 °C until used. The titre of the vaccine was $10^{4,5}$—$10^{6,5}$ $TCID_{50}$/ml and it was applied intranasally.

*Virus neutralization test.* This was carried out in secondary swine thyroid cell cultures with serial serum dilutions.

The milk whey was separated by clotting. Serum and whey samples were inactivated for 30 minutes at 56 °C. Equal volumes of virus containing 100 $TCID_{50}$/0.1 ml was added to each dilution and the mixtures were incubated at 37 °C for one hour. Finally 2—3 tube cultures were inoculated with 0.2—0.2 ml of each dilution. The test was finally read five days p.i.

### *Innocuity tests*

The CKp strain was carried through 14 passages in pig kidney cell cultures and subsequently through 27 passages in swine thyroid cell cultures. The 42nd passage of the strain was tested for innocuity on SPF piglets a few days old (CSONTOS and SZENT-IVÁNYI, 1971) and on a susceptible sow, which was given intranasally 5 ml undiluted tissue-culture virus immediately before farrowing.

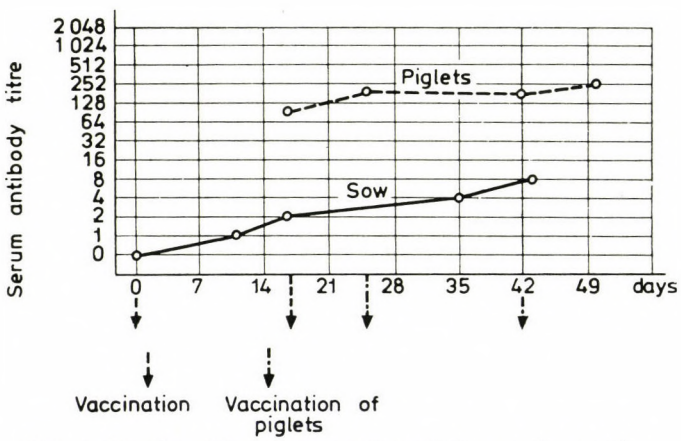


*Fig. 1.* Results of innocuity testing of the vaccine strain

The sow showed no symptoms after vaccination and out of the 11 piglets born 2 days later, only one had a slight diarrhoea for one day. Blood samples were taken from the sow and her litter at regular intervals to check the antibody level. The results are shown in Fig. 1. The appearance of antibodies in the blood of the piglets indicates that the virus was excreted by the sow.

The vaccine was tested in two isolated herds. Seventy-seven sows were vaccinated during the second term of pregnancy. The vaccinees did not show symptoms.

### *Immunization experiments*

The vaccine trial was carried out in two sow herds in which TGE had not occurred. The susceptibility of the sows was confirmed by the serological examination of prevaccination blood samples. In the first herd 34 of the 70 sows were vaccinated. Those which were due for farrowing within 3 weeks were given a single dose, the others received two doses at a 3-week interval. The farrowing of the twice-vaccinated sows began two weeks after the second dose and lasted for another two weeks. Blood and colostrum samples were taken several times from the greater part of the vaccinees. The appearance of antibodies and titre changes are shown in Figs 2a and 2b.

In the second herd 5 of 76 sows were vaccinated on a single occasion (Fig. 3a), 20 twice at a 2-week interval (Fig. 3b) and 18 twice at a 6-week interval (Fig. 3c).

### *Challenge experiments*

In the first experiment, piglets of two sows vaccinated on a single occasion 3 weeks before farrowing were left with the mother animals for 3 and 4 days, respectively; then two piglets from each litter were taken to the laboratory for challenge infection. Two other susceptible piglets were also infected simultaneously with the former four. One sow (No. 119) had serum and colostral whey antibody titres of 1 : 32 and 1 : 2 respectively, the other (No. 16) had respective titres of 1 : 2 and 1 : 4 at farrowing.

One ml of the standard virulent virus was applied intranasally for challenge infection. The intestinal flora of the piglets was normal at challenge.

Two days after challenge the two controls and the piglets of sow No. 16 had a profuse diarrhoea and also the piglets of sow No. 119 had a softer faeces than normally. On the third day all piglets were similarly ill, some of them even vomited. Deaths occurred from the 6th day on. Both control animals and one piglet of sow No. 119, died, the others began to improve and slowly recovered.

Four additional challenge experiments were performed so that the sows were taken to the institute for farrowing and the different litters were infected at various points of time after birth. Litters of small size were completed by piglets of similar age, from similarly vaccinated sows. After challenge, 2 or 3 piglets were isolated from the rest of the litter and were reared artificially to serve as controls.

The course of the challenge experiments was not undisturbed. In one case intercurrent infection by *E. coli* and even *Haemophilus* (Fig. 4), in another case an aetiologically still unclarified infectious disease (Fig. 7) rendere the evaluation difficult.

The results are summarized in Figs 4, 5 and 6, which show fluctuation

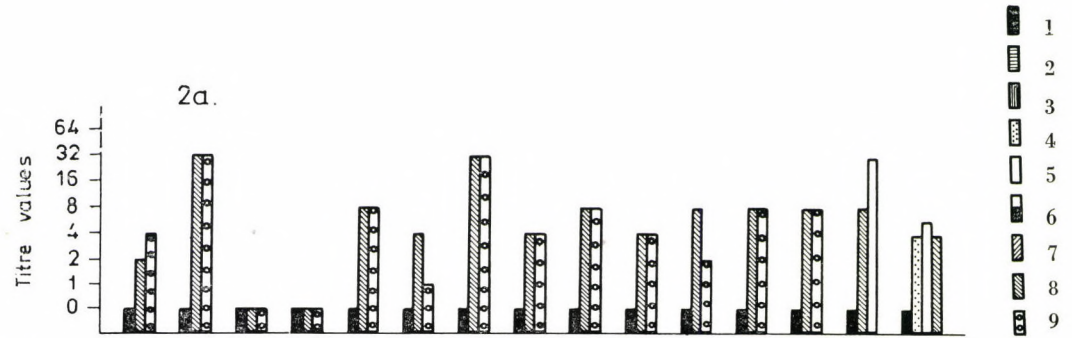


*Fig. 2a.* Dynamics of antibody response to TGE virus. Sows vaccinated with a single dose. Serum and colostral whey titres. Explanation to Figs 2a—3c: 1, Serum titre before vaccination; 2, Serum titre 10 days after vaccination; 3, Serum titre 14 days after vaccination; 4, Serum titre 21 days after vaccination; 5, Serum titre 28 days after vaccination; 6, Serum titre 6 weeks after vaccination; 7, Serum titre 7—8 weeks after vaccination; 8, Serum titre at farrowing; 9, Colostral whey titre at farrowing

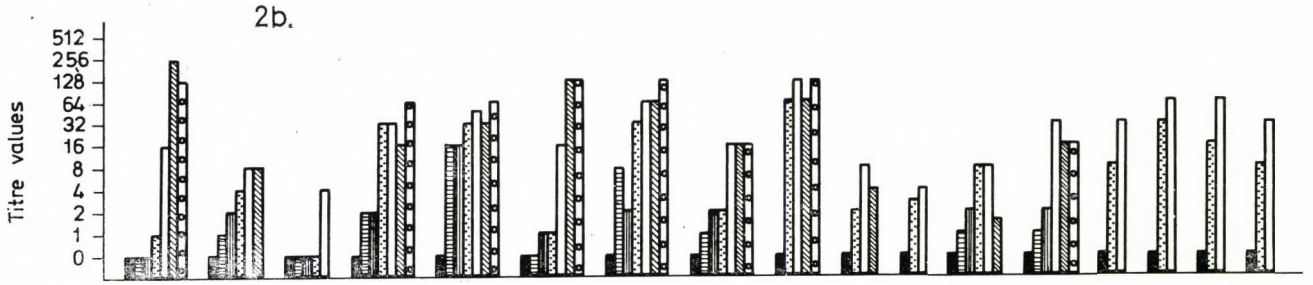


*Fig. 2b.* Dynamics of antibody response to TGE virus. Sows vaccinated twice at a 3-week interval. Serum and colostral whey titre

in the antibody titre in the blood and milk of the sows and in the blood of the piglets as well as in the time and results of challenge infection.

Unlike the first challenge experiment, in the other four the infectious material (suspension of standard virulent virus) was applied by smearing the sows' abdominal region and teats.

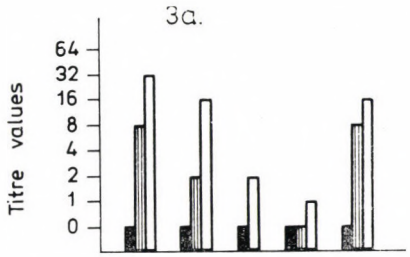


*Fig. 3a.* Dynamics of antibody response to TGE virus. Sows vaccinated with a single dose Serum titres.

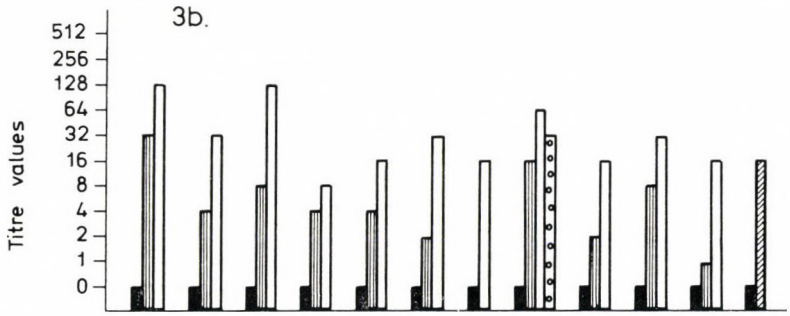


*Fig. 3b.* Dynamics of antibody response to TGE virus. Sows vaccinated twice at a 2-week interval. Serum and colostral whey titres.

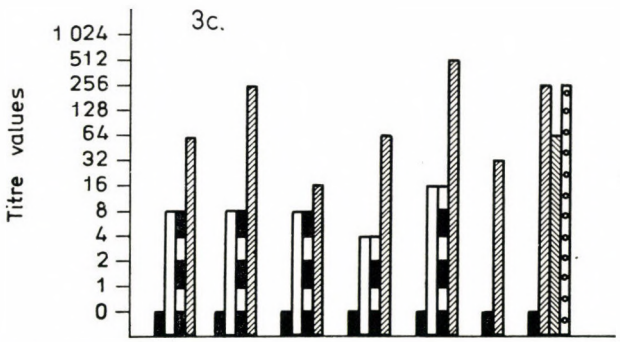


*Fig. 3c.* Dynamics of antibody response to TGE virus. Sows vaccinated twice at a 6-week interval. Serum and colostral whey titres.

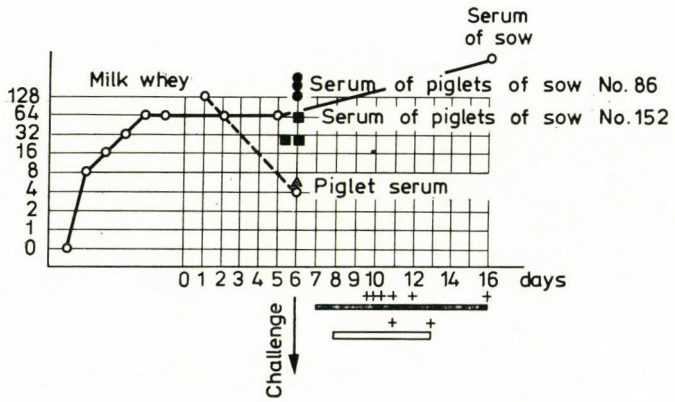


*Fig. 4.* Challenge infection of the piglets of sow No. 106 vaccinated twice at a 3-week interval. Explanation to Figs 4–7: diarrhoea — ■ piglets; □ controls; ▨ sows; + loss

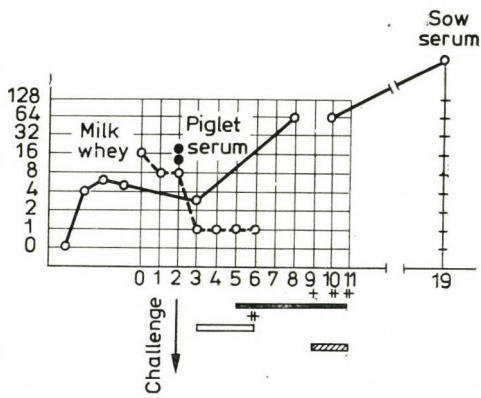


*Fig. 5.* Challenge infection of the piglets of sow No. 134, vaccinated on a single occasion

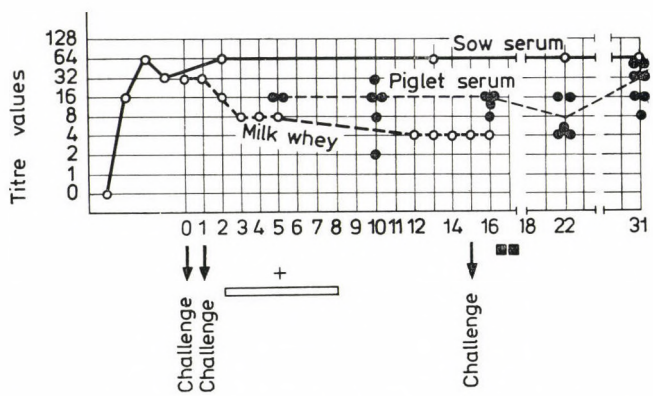


*Fig. 6.* Challenge infection of the piglets of sow No. 280 vaccinated twice at a 2-week interval

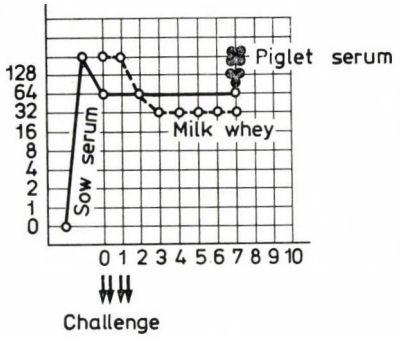


*Fig 7.* Challenge infection of the piglets of sow No. 74 vaccinated twice at a 6-week interval

In the 4th infection experiment, the piglets were reinfected intranasally with 1 ml of the standard virulent virus suspension on the 15th day, to challenge the immune response developed under colostral protection (Fig. 6F).

## Discussion

On the basis of the favourable results obtained in experiments on SPF piglets (CSONTOS and SZENT-IVÁNYI, 1971), the vaccine was tested for innocuity and efficacy in two herds of susceptible pregnant sows. Potency testing required a challenge infection, because spontaneous TGE did not occur in either herd.

The titre of the vaccine was $10^{4,5}$—$10^{6,5}$ $TCID_{50}$/ml; thus with the applied dose each sow was given 150,000 to 15,000,000 TCID units of the vaccine virus.

The vaccine proved to be innocuous. All the sows remained without symptom after vaccination and pregnancy took a normal course. Sow No. 134 proved to be infected by SMEDI virus. Only a single sow, which had been vaccinated two days before farrowing, was found to excrete the virus. Except for this single case, the untreated sows and the growing pigs housed together with the vaccinees did not develop serum antibodies.

Specific antibodies appeared in the serum 10 days after vaccination. The titre increased gradually for four weeks and remained at the final level for at least two months. The antibody level of the milk, which is essential for the protection of the piglets, corresponded roughly with the serum titre of the sow at farrowing. It did not change for two days, then decreased to a half to one-eighth of the birth level during the subsequent 2—3 days and remained at that level for at least 3 weeks. The degree of protection of the newborn piglets depends on the amount of antibody in the milk and on the time of the infection. If the antibody level is high and the sow has abundant milk, the piglets born in the infected environment remain healthy and acquire an active immunity under the protection of the colostral antibodies (Fig. 6).

The piglets of the immunized sows developed TGE when infection occurred after the second postnatal day or when the milk supply was discontinuous and/or insufficient (Figs 4 and 5).

After the intake of colostrum, the serum antibody titres of the piglets roughly corresponded to the titre of the sow's milk on the first or second day. The serum titres persisted at the same level for 1—2 weeks. However the maternal antibodies of the piglets' serum play no role in protection. The only way of disease prevention is the inhibition of virus penetration into the intestinal epithelial cells. This can be achieved if the lumen of the alimentary tract contains antibodies which will neutralize the ingested virus before it has a chance to become adsorbed to the epithelial cells of the small intestine. Thus a continuous supply of maternal antibody is required to prevent TGE and lack or discontinuity of the supply can be held responsible for the susceptibility of those piglets which had been isolated from the sow after the intake of the first colostrum or had been infected at a time when either the antibody content of the milk or the milk production of the sow were low (Figs 4 and 5). This can also account for the failure of earlier trials to protect the newborn piglets with convalescent serum. The present findings accord well with the related observations of HAELTERMANN and HOOPER (1967) and DJURICKOVIC and THORSEN (1970).

It also follows from the results that TGE can occur among the piglets of vaccinated or immune sows if the colostral antibody level is low at the onset of the outbreak.

We made efforts to envolve an optimal vaccination schedule for the sows. As mentioned in the foregoing, either a single dose, or two doses at different time intervals, were administered. The two-dose schedule at an interval of 6 weeks induced the highest antibody response. The logarithms of the antibody titres are shown in Table I.

**Table I**

Average serum antibody titres ($\log_2$ values) of sows vaccinated on different schedules

| Schedule of vaccination | Vaccination interval weeks | Blood samples taken at | | | | |
|---|---|---|---|---|---|---|
| | | 2 | 3 | 4 | 6 | 8 |
| | | weeks after vaccination | | | | |
| Single dose | | | | | | |
| I | — | | 2.93 | | | |
| II | — | 1.75 | | 2.80 | | |
| Two doses | | | | | | |
| III | 3 | 1.00 | 2.70 | 4.40 | | 4.45 |
| IV | 2 | 2.30 | | 4.90 | | |
| V | 6 | | | 3.00 | 3.00 | 6.60 |

The antibody titres obtained by the above vaccination schedule corresponded to those found in the sows' blood after natural TGE virus infection. The first vaccination failed to evoke an immune response in several cases, owing either to technical error or to individual variations in responsiveness. It should be noted that the vaccine must reach the pharyngeal cavity to be effective.

The experiments showed again that young suckling piglets can easily be immunized against TGE. As the vaccine does not cause symptoms if given within several days after birth, active immunization of the suckling piglets may also be taken into consideration in the exposed herds.

## Summary

Vaccination experiments were performed with the TGE virus, CKp strain, which had previously been carried throgh 42 tissue culture passages (15 in pig kidney, 27 in pig thyroid cell cultures). A total of 77 pregnant sows of two herds were vaccinated 3—10 weeks before the expected time of farrowing. Part of the sows were vaccinated on one occasion, part twice, at an interval of 2, 3 or 6 weeks with 5 ml virus suspension on each occasion, by the intranasal route.

The vaccinated animals showed no symptoms. Only a single sow excreted the virus. This had been vaccinated immediately before farrowing. Its piglets had high serum levels of neutralizing antitodies. The vaccine strain proved to be entirely innocuous on application to suckling piglets.

The appearance and titre fluctuations of antibodies in serum and milk were checked by virus neutralization tests several times after vaccination and farrowing. The optimal vaccination schedule proved to be two doses at a 6-week interval, but high serum and colostral antibody levels were found when the two doses were administered at a 2-week interval.

The efficiency of the vaccine was tested by challenge infection of piglets farrowed by the vaccinated sows. It was found that only a continuous supply of antibody-containing colostrum and sow's milk can protect the newborn piglets from TGE in an infected environment. Piglets appropriately supplied with a sufficient amount of maternal antibodies though the milk remained healthy in the infected premises and even acquired an active immunity under colostral protection.

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The address of the authors: Dr. László Csontos, 1149 Budapest, Tábornok u. 2; Dr. Tamás Szent-Iványi, Dr. János Benyeda, 1143 Budapest, Hungária krt. 21, Hungary.

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# VACCINATION EXPERIMENTS AGAINST TRANSMISSIBLE GASTROENTERITIS (TGE) OF SWINE

## II. PERSISTENCE OF POSTVACCINATION TITRES AND THE ANTIBODY RESPONSE TO REVACCINATION

By

J. Benyeda, E. Mocsári and Erzsébet Sághy

Department of Epizootiology, University of Veterinary Science (Head: J. Mészáros), and Central Veterinary Institute (Director: A. Ványi), Budapest

(Received May 9, 1973)

Experimental vaccination of sows in two herds susceptible to TGE virus were described in a previous paper (Csontos et al., 1974).

This paper is a report on the persistence of the postvaccination antibody titres and on the antibody response to revaccination.

### Material and Methods

These were the same as described in the first paper of this series and in other papers related to the topic (Csontos and Szent-Iványi, 1971; Csontos *et al.*, 1973; Csontos *et al.*, 1974).

#### *Determination of the persistence of the postvaccination antibody levels*

The incidence of TGE in the herds during the six-month period following the basic immunization was excluded by a serological examination of the nonvaccinated control sows. Blood samples were then collected from 45 of the 77 sows of the two experimental herds (A and B) vaccinated six months earlier on four different schedules: either a single dose was administered, or two doses at an interval of 2, 3 or 6 weeks (Csontos *et al.*, 1974) and antibody levels were determined by virus neutralization tests.

#### *Determination of the antibody responses to revaccination*

The 45 sows of herds A and B were divided into three groups. Sows of group I (15 animals) were not revaccinated to serve as controls, those of group II (16 animals) were revaccinated once on the day of blood sampling to deter-

mine the postvaccination antibody levels and those of group III (14 animals) were revaccinated twice, viz., on the day of blood sampling and six weeks later. Four and ten weeks after revaccination, blood samples were repeatedly collected from the sows of all three groups and the sera were examined for antibody level by virus neutralization tests.

## Results

The *persistence of the postvaccination antibody levels* is shown in Figs 1 and 2. Sows of herd "A" (Fig. 1) had been vaccinated half a year earlier either on a single occasion, or twice at an interval of 3 weeks. Sows of herd "B"

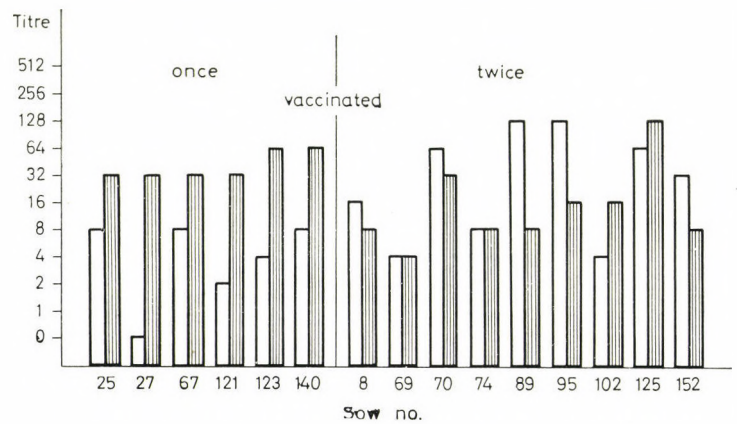


*Fig. 1.* Persistence of antibody titres in sows of herd "A", vaccinated once or twice at a 3-week interval. □ titre immediately after vaccination; ▨ titre 6 months after vaccination

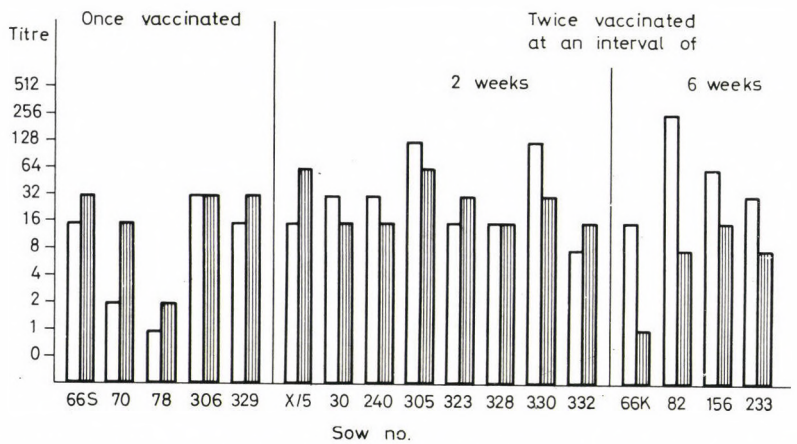


*Fig. 2.* Persistence of antibody titres in sows of herd "B", vaccinated with one dose or with two doses given at a 2-week or a 6-week interval. □ titre immediately after vaccination; ▨ titre 6 months after vaccination

(Fig. 2) had originally been vaccinated during the same period, either on a single occasion or twice, at an interval of 2 or 6 weeks. The empty columns of the figures show the antibody levels attained after basic immunization, whereas the hatched columns show the levels found half a year later.

*Antibody responses to revaccination* can be seen in Figs 3, 4 and 5. In this instance the data of the sows of both herds were pooled. The antibody levels of sows immunized half a year earlier and nonvaccinated again are shown in Fig. 3, those of sows revaccinated on a single occasion in Fig. 4 and those of sows revaccinated twice at an interval of 6 weeks in Fig. 5. The three columns above each earmark number represent the antibody levels found on the day of revaccination and four and ten weeks thereafter.

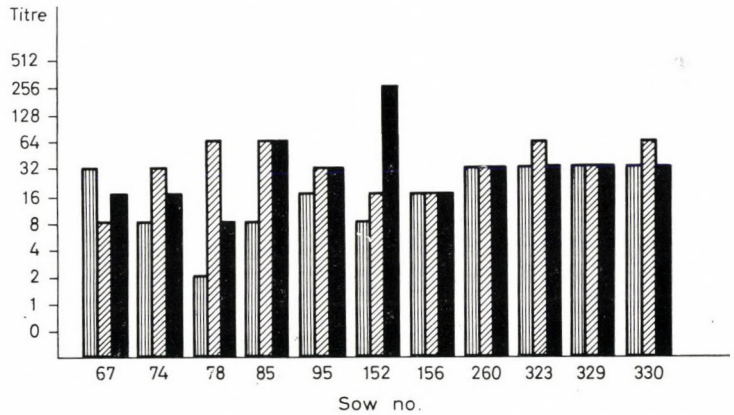


*Fig. 3.* Fluctuation of antibody titres in nonrevaccinated sows. ▥ antibody level on the day of revaccination; ▨ antibody level 4 weeks after revaccination; ■ antibody level 10 weeks after revaccination

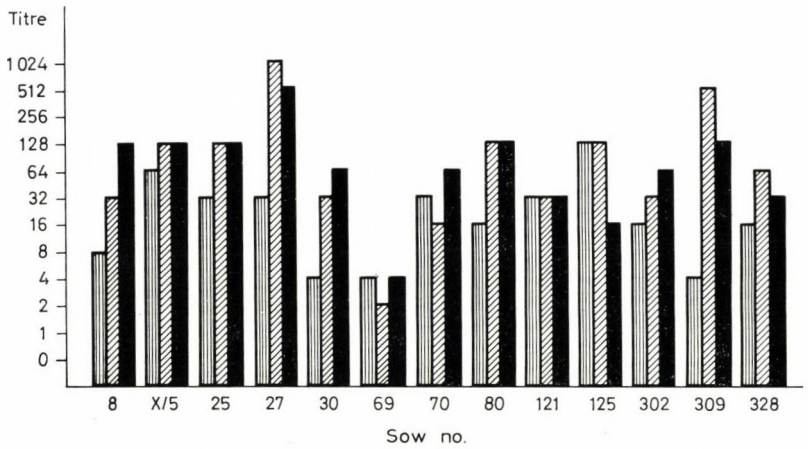


*Fig. 4.* Antibody titres of sows revaccinated once. ▥ antibody level on the day of revaccination; ▨ antibody level 4 weeks after revaccination; ■ antibody level 10 weeks after revaccination

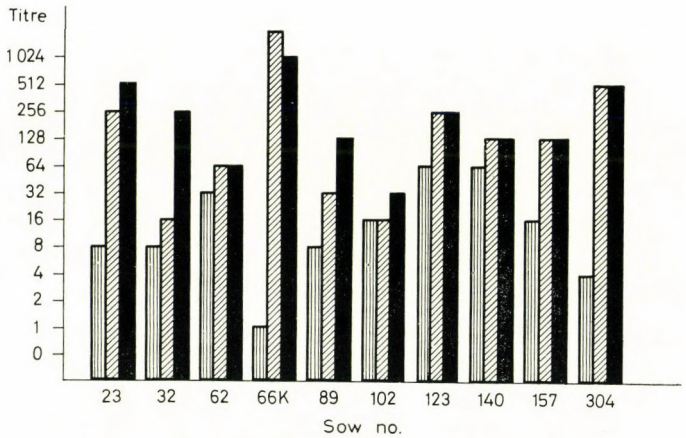


*Fig. 5.* Antibody titres of sows revaccinated twice at an interval of 6 weeks. ▨ antibody level on the day of revaccination; ▨ antibody level 4 weeks after revaccination; ■ antibody level 10 weeks after revaccination

## Discussion

Since, at the time of the closing of the experiment, TGE had not occurred in the two herds, not even among the nonvaccinated susceptible animals, the serum antibody titres of the immunized sows found six months after the basic vaccination were due to the immunization. The titres differed markedly, depending on the schedule of immunization. The mean titres of the sows given a single dose of the vaccine were much higher than immediately after immunization. The titres of the sows vaccinated twice at a 2-week interval persisted at the original level, whereas those of the animals vaccinated twice at an interval of 3 or 6 weeks fell slightly and markedly, respectively. The numerical evaluation of the results is shown in Table I.

**Table I**

Persistence of antibody titres in groups of sows vaccinated at different time intervals

| Sows | $\log_2$ titre | |
|---|---|---|
| | attained after vaccination | 6 months after vaccination |
| Vaccinated on a single occasion | 2.40* | 4.67 |
| Vaccinated twice at an interval of 2 weeks | 4.87** | 4.74 |
| 3 weeks | 4.66*** | 3.77 |
| 6 weeks | 5.75**** | 2.50 |

Explanation: * 4 weeks after vaccination; ** 2 weeks after the second vaccination; *** 1 week after the second vaccination; **** 10 days after the second vaccination

The higher titres fell more markedly than the lower ones. It is of interest that the antibody levels of the sows vaccinated on a single occasion did not decrease in any case; a single sow had an unchanged titre, all others showed an increase. Further investigation would be necessary to discover the cause of this phenomenon.

The degree of titre increase after revaccination depended on the pre-revaccination antibody level. Lower antibody titres increased more markedly than the higher ones. A slight rise in titre was also observed in sows not re-vaccinated six months following the basic immunization. Immune response was not notably better in the case of repeated revaccinations than in the case of a single one. The results are summarized in Table II.

**Table II**

Changes of antibody titre after revaccination

| Sows | $\log_2$ titre | | |
|---|---|---|---|
| | on the day of revaccination | 4 weeks after revaccination | 10 weeks after revaccination |
| Non revaccinated | 3.91 | 5.09 | 4.91 |
| Revaccinated once | 4.15 | 6.00 | 6.00 |
| Revaccinated twice | 3.60 | 5.90 | 7.60 |

The aim of the experiments was to find the optimal vaccination schedule which would ensure the persistence of the antibody titre at a satisfactorily high level for a long time after immunization. Two doses of vaccine administered at a 2-week interval seemed to meet this requirement, because fairly high average antibody levels appeared after the basic immunization and these changed but little within half a year. In spite of this, the fact remains, that two doses of vaccine, given at an interval of six weeks, induced a higher antibody response than the aforementioned schedule. As outbreaks of TGE occur only in a given period of the year and as a firm protection of the newborn piglets can only be ensured by a high antibody contents of the colostral and sow's milk, we have the intention to carry out a more detailed examination of the persistence of antibody levels obtained after the six-week immunization schedule and to determine the point of time when their decrease begins.

Examinations after revaccination showed that the antibody levels persisting six months after the basic immunization could be brought to a high mean level by a single revaccination. Sows with this high antibody level provide a firm protection for their piglets born in the critical period of TGE outbreaks through the high antibody contents of colostrum and milk.

## Summary

Sows experimentally vaccinated against TGE half a year earlier were examined for the persistence of serum antibody titres and for titre changes after a subsequent revaccination.

The sows originally vaccinated once showed an average rise by 2.27 $\log_2$ units compared to the original mean titre. The antibody levels of animals vaccinated twice at an interval of two weeks showed but slight changes over the period of six months. The titres of sows twice vaccinated at 3- and 6-week intervals fell by 0.89 and 3.25 $\log_2$ units, respectively. Higher postvaccination titres fell more markedly than the lower ones.

Titre rise after revaccination depended on the antibody level existing six months after basic immunization. Low titres rose more markedly than the higher ones. The results obtained by a single or by repeated revaccinations did not notably differ from one another.

It is concluded that the persistence of the postvaccination antibody level can best be achieved with two doses of vaccine administered at an interval of two weeks and that revaccination with a single dose after half a year gives rise to a fairly high mean antibody level.

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The address of the authors: Dr. János Benyeda, Dr. Endre Mocsári, Erzsébet Sághy, 1143 Budapest, Hungária krt. 21, Hungary.

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# STUDIES ON THE COCCIDIA OF THE DOMESTIC CAT. *ISOSPORA NOVOCATI* SP. N.

By

L. PELLÉRDY

Department of Parasitology (Head: Prof. T. KOBULEJ), University of Veterinary Science, Budapest

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Remarkably enough, the coccidia of the domestic cat are not known in full detail, although this animal lives in the immediate surroundings of man. The best known feline coccidium *Isospora felis* (Wasielewski, 1904) Wenyon, 1923 is probably the only of its kind which can be differentiated with certainty from all other isosporas of the cat and dog, due to the characteristic shape and unusually large size of its oocysts. The characterization of the other feline coccidia is uncertain, because species differentiation by oocyst morphology is difficult or, even impossible.

Next in frequency to *Isospora felis* is *Isospora rivolta* (Grassi, 1879) Wenyon, 1923, but, according to several authors, this is not exclusively a cat coccidium, occurring in other *Felidae* and in the family *Canidae* as well, at least as judged on the basis of oocyst morphology. Since coccidia are generally characterized by a strict host specificity, one cannot help to feel sceptical about the alleged identity of the feline and canine *Isospora rivolta* species.

The less frequent cat coccidium *Isospora bigemina* (Stiles, 1891) Lühe, 1906 was reported to have been found in the faeces of the dog, common red fox *(Vulpes vulpes)* and pole cat *(Mustela putorius)* as well. The alternately positive and negative results of several experimental infections rendered the validity of the species uncertain until it was demonstrated that the spherical or elliptic oocysts with a maximum diameter of 10—14 $\mu$ are actually *Toxoplasma* oocysts, at least in the feline host. Certain observers classified also larger oocysts, 18—20 by 14—16 $\mu$ in size, as *Isospora bigemina;* these oocysts contain two sporoblasts or sporocysts when passed and have a thin wall which soon becomes puckered and associates closely with the sporocysts. WENYON himself (1926) wondered about this finding and concluded that more than one species had been classified as *Isospora bigemina* in the descriptions.

RAILLIET and LUCET (1891) described *Coccidium bigeminum* var. *cati* from cat supposing *C. bigeminum* was specific parasite of the dog.

Apart from the foregoing three *Isospora* species, several eimeriae have been described from the domestic cat. Out of them *Eimeria cati* Yakimoff, 1933, was reported to have been found in the faeces of the jungle cat *(Felis chaus)* and *Eimeria felina* Nieschulz, 1924, in that of the lion *(Panthera leo)*. Probably these eimeriae were not genuine parasites of the above hosts, but belonged specifically to other animals, which had been eaten by the carnivores, and these passed the "foreign" oocysts with their faeces.

Apart from the above coccidia no other *Eimeria* or *Isospora* species have been described from the domestic cat.

## Experimental

In the course of the *Toxoplasma* investigations on cats and mice, feline coccidia were studied in great detail, including the new species whose description is forthcoming. The positive results of six infection experiments performed

on cats with sporulated oocysts of the new species clearly showed that the latter is in fact a parasite of the domestic cat. Unfortunately, its endogenous development could not be studied for lack of pure strains and coccidium-free cats.

*Isospora novocati* sp. n.*

Synonym: *Isospora rivolta* (Grassi, 1879) Wenyon, 1923, pro parte.

The oocysts are round or subspherical; round oocysts are 24—30 $\mu$ (average: 27 $\mu$) in diameter, the short elliptic ones measure 22—30 by 21—27 $\mu$ (average: 26 by 24 $\mu$). The wall is smooth, light-coloured, 1—1.5 $\mu$ thick and bilayered. Being not strong, it puckers in hypertonic solutions in about one hour. The oocyst wall bears no micropyle.

The oocysts are passed in an unsporulated state. The sporont is found in an eccentric position within the oocyst wall. Sporulation takes 1—2 days at room temperature.

On sporulation, the sporocysts are symmetrically arranged, parallel to the longitudinal axis of the oocyst. They are broad elliptic, 15 by 12 $\mu$ in size and do not bear a Stieda body. Neither polar body, nor oocyst residuum is present in the oocyst. A coarsely granular sporocystic residuum lies between the banana-shaped sporozoites.

In infection experiments performed at different times on a total of six young, about 2 months old, cats the oocysts appeared in the faeces 5 days after the single infection and their number increased rapidly from the 7th day on. The infection lasted 5—6 weeks in the cats which could not fully be protected from reinfection, suggesting that the immune response was either weak or absent.

After the closing of the experiment, the cats were killed. The presence of oocysts in the ileum showed that the endogenous development of *Isospora novocati* takes place in the anterior portion of the small intestine, either in the duodenum or in the jejunum.

*Host specificity*. The negative results of infection experiments on four 2-month-old puppies, free from any kind of coccidium, showed that *Isospora novocati* is not transferable to dog. The experimental infection was followed up by coprological examinations, first daily, later at intervals of 3—4 days, over an observation period of one month.

*Pathogenicity*. The experimentally infected cats passed oocysts in increasing numbers from the 6th day on. The cats developed a diarrhoea 1—2 days before the excretion of oocysts reached maximum, but the symptoms subsided with the decline of oocyst discharge.

* As DUBEY, MILLER and FRENKEL (1970) suggested the name *Isospora cati* — although only as a *nomen dubium* — for the designation of RAILLIET and LUCET's (1891) *Coccidium bigeminum* var. *cati*. I had better omit this denomination to prevent confusion.

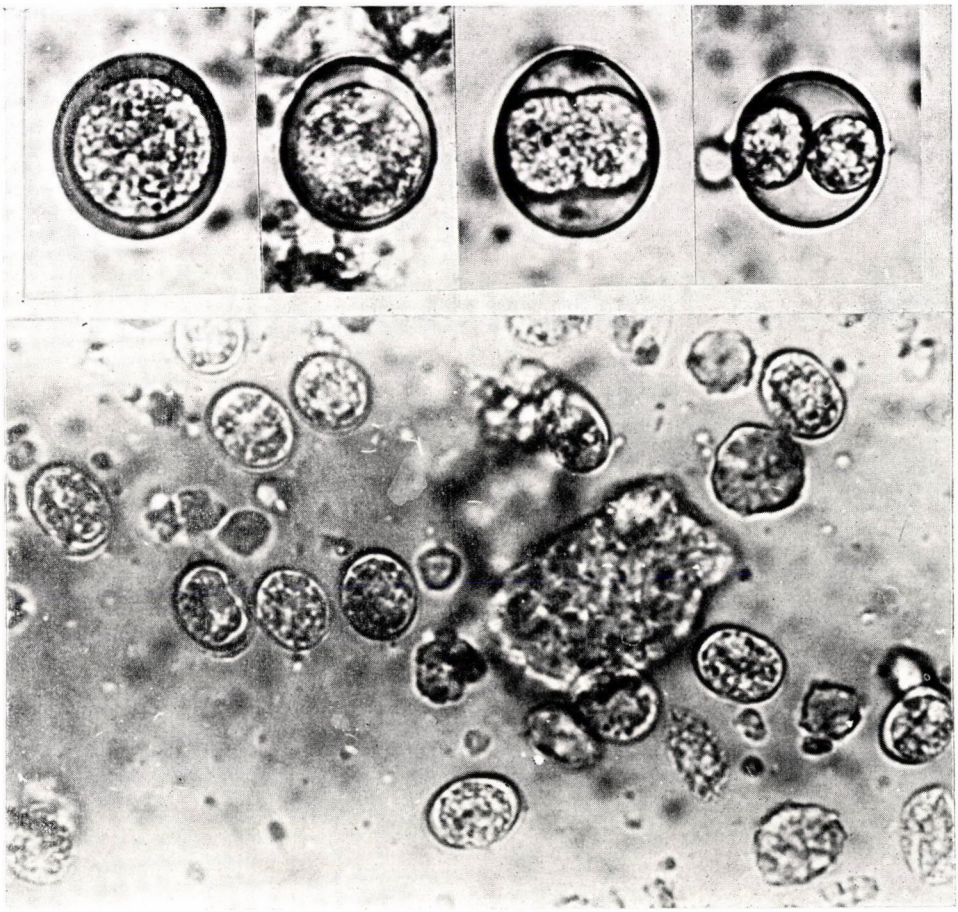

*Fig. 1.* Top: Four *Isospora novocati* sp. n. oocysts in different stages of sporulation. ×1000; Bottom: *Isospora bigemina* (Toxoplasma) oocysts shown for comparison. ×1000

## Discussion

The oocysts of *Isospora felis* are almost twice as large as those of *Isospora novocati*, whereas those of *Isospora bigemina* (= *Toxoplasma*) are much smaller, thus only the differentiation from *Isospora rivolta* is explained here in detail. *Isospora rivolta* had originally been found in, and was described from, the dog. WENYON (1926) gave the dimensions of the oocysts as 20—25 by 15—20 $\mu$, and described them as oval, with a smooth, 0.8 $\mu$ thick wall consisting of a single layer. Several authors, including myself, observed that the wall of the canine *Isospora rivolta* oocysts soon becomes puckered and associates closely with the sporocysts, whence the oocysts assume a dumbbell-shape. When the cyst wall disrupts, free sporocysts can appear in the faeces.

The oocysts of the feline coccidium regarded as *Isospora rivolta* differ from the corresponding stages of the homonymous canine coccidium in several morphological features. The oocysts of the feline *Isospora* are round or short elliptic rather than oval and do not taper at one end. Also, they are larger than the canine *Isospora rivolta* oocysts and their wall is apparently stronger, as dumbbell-shaped oocysts are rarely encountered in cat faeces. Similar observations were made by SHAH (1970), who, however, still regarded the "ellipsoid to somewhat ovoid" oocysts from the cat 21—28 by 18—23 $\mu$ (mean: 25 by 21.1 $\mu$) in size, as *Isospora rivolta*.

The negative results of a few infection experiments have affirmed the host specificity of feline and canine *Isospora* species. *Isospora rivolta* had originally been found by GRASSI in the dog and later observers classified the *Isospora* of the cat into the same species, merely on the basis of morphological similarity. MAHRT (1965; cit. SHAH, 1970) failed to infect three young cats with oocysts originating from dog. SHAH, although he did not regard MAHRT's results as conclusive, infered from them that the *Isospora rivolta* strains occurring in cats are not the same as those occurring in dogs.

Among the earlier studies mention should be made of the negative cross infection experiments reported by BÖHM (1923). This author failed to transfer *Isospora* species from dog to cat and *vice versa*.

Apart from *Isospora rivolta*, *Isospora felis* was used by several investigators for transinfection experiments on dogs, with invariably negative results. Taking into consideration the shape and size of *Isospora felis* oocysts, which characteristically differentiate them from the corresponding stages of other feline isosporas, there is reason to suspect that the sporadic positive results of transinfection experiments may have been based on erroneous observations. The oocysts of the canine *Isospora rivolta* and of the feline *Isospora novocati*, described in this paper, scarcely differ morphologically and their differentiation requires tenacious work. As to earlier studies it can be said (PELLÉRDY, 1965) that the conditions of experiments were not quite what they should have been; insufficient consideration was given to the experimental animal's possible immunity and in evaluating the results of the experiments exaggerated importance was attached to the outcome of observations in the field.

In the present experiments, *Isospora rivolta*-like oocysts from the cat were not transferable to four young dogs, which had been free from any coccidia prior to experimental infection. In view of this finding and on the basis of the foregoing considerations it seems justified to regard the similar oocysts occurring in cat and dog as stages of two independent species. Presumption of different strains or subspecies would be unfounded in the case of coccidia. As *Isospora rivolta* was originally described from the dog, it seems correct to classify the similar, but by no means identical, cat coccidium as an independent species, under the name *Isospora novocati*.

## Summary

A coccidium of the domestic cat *(Felis catus)*, is described under the name *Isospora novocati* sp. n. It resembles to a certain degree the *Isospora rivolta* of the dog, but has several well defineable differentiating features. The oocysts are round or elliptic; the round ones are 24—30 $\mu$ in diameter, the elliptic forms measure 22—30 by 21—27 $\mu$. Sporulation takes 1—2 days at room temperature, the prepatent period is 6 days. Experimental infection of young dogs with the sporulated oocysts failed. A massive experimental infection elicited a temporary diarrhoea in cats.

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The address of the author: Dr. László Pellérdy, 1071 Budapest, Damjanich u. 56, Hungary.

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# A STUDY OF GASTRIC ACID PRODUCTION IN PIGS IN RELATION TO EARLY WEANING

By

F. KUTAS and J. SZABÓ*

Department of Physiology (Head: † Prof. A. KEMÉNY), University of Veterinary Science and Phylaxia Institute of Veterinary Biologicals and Feedstuffs (Director: J. MOLNÁR), Budapest

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Hydrochloric acid is an essential factor in the peptic digestion of mammals. Its main role is the activation of pepsinogen in the stomach. In addition, hydrochloric acid acts as an antiseptic agent: the acid pH is unfavourable to the growth of microorganisms in the stomach. Consequently, the gastric fermentation of carbohydrates as well as the putrefactive processes are inhibited. The gastric acid barrier of the stomach also aids in the prevention of body infections. Considering the role and importance of hydrochloric acid in the organism, it is quite understandable that several papers have appeared in the past concerning the inadequacy of hydrochloric acid secretion in the stomach of sucking pigs.

CUNNINGHAM and BRISSON (1957) recorded pH values of 1.85—3.20 in the stomach of newborn pigs. WHITE *et al.* (1972) studied the postnatal gastric acid secretion in pigs. The lowest pH in the stomach content was found at birth: pH 3.0—3.5. According to their suggestion, this relative acidity can be explained with intrauterine factors: the maternal gastrin crosses placenta and stimulates foetal stomach. At parturition, this supply of gastrin caeses and pH increases. At 3 days of age, the mean value for stomach pH is above 5.0. The pH of the stomach content of 2- to 3-week-old nursing pigs was found in all cases above 3.5 (CUNNINGHAM and BRISSON, 1957). Others reported similar values: stomach pH of 6-hour fasted sucking pigs was in average more alkaline than pH 3.4 at all ages up to 5 weeks (LIU *et al.*, 1954; WALKER, 1959).

Judging from the results of the foregoing observations it appears that at the period of early weaning the pH of stomach content is between 3.5 and 4.5. Since the optimum pH of pepsin activity is approximately 2, the activity of enzyme is very slight at these pH values.

Several workers studied the origin of $H^+$ ions found in the stomach of sucking pigs. Since hydrochloric acid appears only after 30 days of age, the authors believed that the nature of the food and the microflora of the stomach were responsible for the gastric acidity in early life (KVASTNITSKIY and BAKEYEVA, 1940). The source of $H^+$ ions is mainly lactic acid, the product of the bacterial fermentation of carbohydrates in the achlorhydric stomach (CRANWELL *et al.*, 1968; NOAKES *et al.*, 1968). Significant quantities of lactic acid were detected in the stomach contents of sucking pigs at 1 week of age (FRIEND *et al.*, 1963). Hydrochloric acid secretion sufficient to produce an acidity optimal for the activation of pepsinogen does not occur until 20—30 days of age (KVASTNITSKIY, 1953). No free hydrochloric acid was detected in the stomach contents either by titration with Topfer's reagent or by using Gunzberg's test from birth to 5 weeks of age (WALKER, 1959). However, hydrochloric acid secretion may occur earlier when cereals are given after early weaning (STAROVOYTOV, 1956).

In view of the foregoing observations, it can be concluded that despite numerous excellent studies on sucking pigs, only limited information is avail-

* With the technical assistance of Lidia KOVÁTS

able on the gastric acid secretion of weaned animals. However, the application of early weaning as a practical aspect of pork production needs physiological data concerning the post-weaning period. In order to furnish the missing informations, the present work was undertaken to study the effect of weaning at 1 week of age on gastric acid secretion. In the first part of our work the pH of natural stomach samples were compared in sucking and weaned pigs. In addition, the fractional method of gastric analysis was applied in both groups to establish whether the stomach of the pigs can produce any hydrochloric acid at all.

## Materials and Methods

### *Experimental animals*

A total of 109, mostly cross-bred piglets with ages ranging from 3 to 27 days were used in the investigations. 69 animals nursed their dams throughout the experimental period, 40 pigs after weaning at 1 week of age ate self-fed dry rations (crude protein: 25%, fat: 19.2%, ash: 6.7%, N-free extract: 42.9%). The weaned pigs were housed in pens in groups of five.

### *Experimental procedure*

*Removal of natural stomach contents.* The samples were taken always in the morning hours between 8 and 10 o'clock. A soft rubber tube, 3 to 6 mm in internal diameter, with a rounded tip and 4—5 lateral holes, was passed into the stomach and 5—10 ml of content was withdrawn by suction with a syringe. The piglets were allowed to suckle or feed *ad libitum* before taking samples.

*Stimulation of gastric acid secretion.* 6 to 8 hours fasted pigs were used. Prior to carry out the histamine test, the stomach was emptied by suction and was washed with about 40 °C warm tap-water. The washing fluid was rapidly withdrawn. The same rubber tube was used as at the removal of the natural stomach contents. After 15 minutes, Peremin (histamine dihydrochloride, Chinoin, Budapest) was administered by subcutaneous route in dose of 0.03 mg/kg body weight. The time at which histamine injection was given was noted as the zero time. At intervals of 15 minutes from this time, samples of gastric content were withdrawn from the stomach. The samples were stored at —15 °C.

### *Analytical procedure*

The stomach content was centrifuged and the supernatant was used for laboratory determinations. Since in most cases only a small amount of liquor was obtained, the use of a micro-method was necessary. The pH of the sam-

ples was measured in a micro-electrode system which consisted of Radiometer's pH-meter PHM27 and combined electrode E5021. For the determination of pH only 0.1 ml of supernatant was needed. When the pH was below 3.0 it was considered as sample with the presence of free hydrochloric acid.

## Results

*The pH and free hydrochloric acid in the natural gastric content.* The pH of stomach content of 42 sucking pigs was measured at the pre-weaning period. The mean pH was found $4.06 \pm 0.089$. 20 pigs were weaned at 7 days of age and gastric contents were obtained at 5 days intervals until the age of 21—22 days. In this way 39 samples were collected with the mean pH of $4.43 \pm 0.145$. Sucking animals of the same age (12—22 days) were also studied. In this case the mean pH of gastric samples was found $4.92 \pm 0.184$. In the majority of samples no free hydrochloric acid was present. Only 3 stomach contents taken from weaned pigs contained free hydrochloric acid.

*Stimulated gastric secretion with histamine.* For the purpose of presentation of our results, the secretory responses of pigs were divided into 3 groups. With regard to the special features of gastric secretion in the young pig, the following categories were formed:

(a) *Achlorhydria* (absolute lack of hydrochloric acid secretion). After histamine injection none of the samples contains free hydrochloric acid.

(b) *Hypochlorhydria* (incomplete secretion of hydrochloric acid). In 1 or 2 of samples can be found free hydrochloric acid.

(c) *Chlorhydria* (normal secretion of hydrochloric acid). All or 3 of samples contain free hydrochloric acid.

**Table I**

Comparison of hydrochloric acid secretions after histamine stimulation in sucking and weaned pigs of the same age

| Type of acid secretoon | Number of types of acid secretion at | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 11—12 days of age | | 16—17 days of age | | 21—22 days of age | | 24—27 days of age | | Total | |
| | S | W | S | W | S | W | S | W | S | W |
| Achlorhydria | 4 | 3 | 4 | 2 | 3 | 1 | 2 | 1 | 13 | 7 |
| Hypochlorhydria | — | 1 | 1 | 1 | 2 | 1 | 2 | — | 5 | 3 |
| Chlorhydria | 1 | 1 | — | 2 | — | 3 | 1 | 4 | 2 | 10 |

S, sucking; W, weaned

The patterns of pH changes are presented graphically in Fig. 1.

In the first part of our work, the gastric acid secretion was studied at the pre-weaning period. 15 sucking pigs of 3 to 7 days of age were stimulated

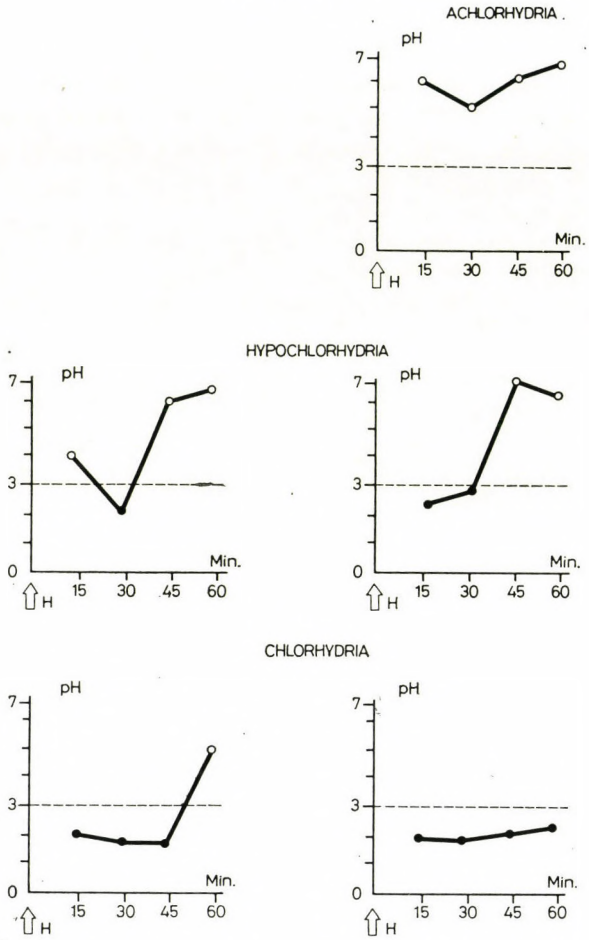


*Fig. 1.* The figure illustrates the different types of achlorhydric, hypochlorhydric and chlorhydric pH curves. H, histamine injection

with histamine injection. In 12 of the animals studied there was no free hydrochloric acid in the gastric juice (achlorhydria). The gastric secretion of 3 pigs was of hypochlorhydric type, since only in the gastric samples taken at 15 minutes could be detected free acid.

The histamine test was also performed on weaned pigs at 5 days intervals. At the same time, sucking piglets of the same age served as controls. The results shown in Table I indicate that most pigs in the sucking group showed gastric secretion of achlorhydric type, and on the other hand, the greatest number of pigs weaned at 1 week of age secreted free hydrochloric acid (chlorhydria).

The relative differences observed in the gastric secretion of unweaned and weaned pigs are also demonstrated in Fig. 2. In this figure the pH values of all gastric samples (80—80) obtained after histamine injection are sum-

marized. The curves of each group give informations about the percentage distribution of stomach samples in the different pH-intervals. It can be established that the greatest part of gastric samples taken by weaned pigs showed pH values between 1 and 2. In contrast, in case of sucking animals, the peak of the curve was between pH 7 and 8.

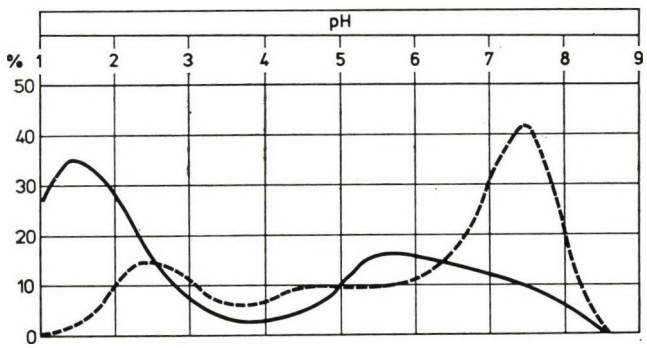


*Fig. 2.* Percentage distribution of pH values in the gastric juice of unweaned (— — — —) and weaned (————) pigs with ages ranging from 11 to 27 days, following histamine injection. See text for discussion

## Discussion

The stimulation of gastric secretion was performed in 35 sucking pigs. Only 2 animals (6 per cent of total) responded with hydrochloric acid production following histamine injection. In 71 per cent of cases (25 pigs) hydrochloric acid was not detected in the gastric samples. These results indicate that during the first month of life the gastric secretion of sucking animals is predominantly of achlorhydric type. The lack of hydrochloric acid production has no deleterious effect on protein digestion, since the pepsin activity of the stomach tissue is very low during the first weeks (HARTMAN *et al.*, 1961). Accordingly, there is no need for the activation of enzyme by $H^+$ ions. The gastric proteolysis is minimal and the small intestine is the main site of the proteolytic processes. The young pig is capable of producing relatively high amounts of trypsin (LUCAS, 1962). During the first weeks of age, trypsin appears to be the main protein-splitting enzyme. The efficiency of protein digestion can be characterized with the high value of apparent digestibility of the sows' milk protein which is about 98 per cent until 5 weeks of age (LIVINGSTONE *et al.*, 1960). Consequently, in spite of achlorhydria present in sucking pigs, there is no disorder in the digestion of proteins. However, the achlorhydria is not indifferent from the point of view of the organism, since the lack of gastric acid barrier diminishes the "defensive ability" of the animal against peroral bacterial invasions. Evidence is provided (KVASTNITSKIY, 1953) to indicate

that gastric juice of young sucking pigs has practically no bactericidal character. Significant antiseptic action of gastric juice can be detected only after 40 to 50 days of age.

The situation is quite different in the early weaned pig. From the present studies it is concluded that when the pigs are weaned at 1 week of age, hydrochloric acid is secreted soon after weaning. In most animals chlorhydria was found before the age of 4 weeks.

Considering the above mentioned observations, the question arises why the early appearance of hydrochloric acid secretion does not produce a low pH in the natural gastric contents after weaning. Only slightly lower average pH values were noted in the natural stomach contents of weaned pigs (pH 4.43) than in those of sucking animals (pH 4.92). The relatively small difference can be explained with the great acid buffering capacity of milk and baby starter. It was shown in a previous investigation (KUTAS *et al.*, 1973) that 1 g of dry matter of both sows' milk and baby starter can buffer 4 to 6 ml of 0.1 ml N HCl while the pH is reduced only to 4.0. Calculating with a dry matter intake of 200 g per piglet, this amount can buffer about 1 lit. of 0.1 N hydrochloric acid without diminishing the pH below 4.0.

## Summary

A comparative st udy was made of the gastric acid secretion in weaned *vs.* unweaned pigs, from 1 week through 4 weeks of age. The gastric mucosa was stimulated with subcutaneous histamine injection and the secretory response was studied by measuring the pH of the gastric juice. Most sucking pigs were achlorhydric or hypochlorhydric: no or only slight hydrochloric acid secretion was detected in gastric juice following the administration of histamine. In contrast, the greatest number of piglets weaned at 1 week of age showed hydrochloric acid production after histamine injection.

From the present studies it may be concluded that the period of achlorhydria is significantly shorter in early weaned pigs when it is compared witd that of sucking animals. It was noted that the hydrochloric acid secretion develops relatively fast after weaning. The earlier appearance of gastric acid secretion has beneficial effect on gastric proteolysis and, at the same time, it may promote the prevention of peroral infections.

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Address of the authors: Dr. Ferenc KUTAS, 1078 Budapest, Landler J. u. 2; Dr. József SZABÓ, 1107 Budapest, Szállás u. 3, Hungary.

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# SEROLOGICAL STUDIES OF BOVINE MYCOPLASMAS AND ACHOLEPLASMAS

By

L. STIPKOVITS and LEA VARGA

Veterinary Medical Research Institute (Head: D. DERZSY), Hungarian Academy of Sciences, Budapest

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Several *Mycoplasma* and *Acholeplasma* species [*M. mycoides* subsp. *mycoides* (EDWARD and FREUNDT, 1956), *M. bovigenitalium* (EDWARD, 1947), *M. bovirhinis* and *A. laidlawii* (LEACH, 1967), *M. agalactiae* subsp. *bovis* (HALE, HELMBOLDT, PLASTRIDGE and STULA, 1962), *M. arginini* (BARILE, DEL-GIUDICE, CARSKI, GIBBS and MORRIS, 1968), *M. dispar* (GOURLAY and LEACH, 1970), *M. alkalescens* and *A. modicum* (LEACH, 1973), *M. oculi* (LANGFORD and LEACH, 1973)] and serogroups [Leach's group 7 (LEACH, 1967), Al-Aubaidi's serogroups H, I, K and L (AL-AUBAIDI and FABRICANT, 1971)] have been described among the strains of bovine origin. Some useful cultural and biochemical procedures have been recommended for preliminary identification purposes (ERNØ and STIPKOVITS, 1973) but the final identification of the strains can be performed by serological tests.

The present paper describes serological studies of bovine mycoplasmas and acholeplasmas by means of growth inhibition (GIT) and metabolic inhibition (MIT) tests. These studies were performed as a comparative examination of reference sera some of which had been obtained from Dr. J. G. TULLY (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland U.S.A.), from Dr. H. ERNØ (Institute of Medical Microbiology, FAO/WHO Centre, University of Aarhus, Aarhus, Denmark) and from Dr. R. H. LEACH (Central Public Health Laboratory, Mycoplasma Reference Laboratory, Colindale Av. London, England), as well as hyperimmune sera prepared in our laboratory.

## Methods

*Strains.* The strains used for our studies are listed in Table I. All strains were obtained from the Institute of Medical Microbiology, University of Aarhus, Aarhus, Denmark in dried condition. The ampoules were opened and broth culture was made from each strain, a part of it was dried again, another part was stored as working culture at —20 °C.

*Production of antisera.* Antigens for immunization of rabbits were prepared from cultures grown in medium B (ERNØ and STIPKOVITS, 1973), in which the horse serum was replaced by 1% PPLO Serum Fraction (Difco). The cultures being in late log phase were sedimented at 10 000 X g. (Zuglói Gépgyár) for 40 minutes and the sediment was washed three times in PBS

(pH: 7.2) and resuspended in PBS at 1 : 20 of original volume. Antigens obtained were stored at —20 °C.

*Antiserum production.* Young male New Zealand rabbits of 2 kg weight were used for producing antisera according to the method published by MORTON and ROBERTS (1967). Two hyperimmune sera were prepared with each strain and examined with each strain in both GIT and MIT. For comparison reference sera obtained either from Dr. J. G. TULLY, from Dr. H. ERNØ, or from Dr. R. H. LEACH were used. No reference sera against Al-Aubaidi's serogroups H, I, K and L were available. Serogroups I, H and K were also examined by reference sera against *M. gateae*, *M. gallinarum* and *A. axanthum*.

**Table I**

Type or reference strains of bovine Mycoplasmas and Acholeplasmas

| Species or serogroup | Type or reference strain | Supplied by | Isolated by | Cultured from |
|---|---|---|---|---|
| *M. bovigenitalium* | PG 11 | Aarhus Univ. | EDWARD (1950) | genital tract |
| *M. bovigenitalium* | Kalle HE 1 | Aarhus Univ. | ERNØ (1967) | genital tract |
| *M. bovirhinis* | PG 43 | Aarhus Univ. | HARBOURNE, HUNTER and LEACH (1965) | lung |
| *M. arginini* | G 230 | Aarhus Univ. | MORRIS (1968) | brain |
| *A. laidlawii A* | PG 8 | Aarhus Univ. | LAIDLAW and ELFORD (1936) | sewage |
| *A. laidlawii* B | PG 9 | Aarhus Univ. | LAIDLAW and ELFORD (1936) | sewage |
| *M. mycoides* subsp. *myc.* | PG 1 | Aarhus Univ. | LAIDLAW (1931) | lung |
| *M. agalactiae* subsp. *bovis* | Donetta | Aarhus Univ. | HALE et al (1962) | udder |
| Group 7 (Leach) | PG 50(N29) | Aarhus Univ. | SIMMONS and JOHNSON (1963) | joint |
| *M. alkalescens* | PG 51 | Aarhus Univ. | HUDSON and ETHERIDGE (1963) | nose |
| Group H(Al-Aubaidi) | B 139 P | Aarhus Univ. | AL-AUBAIDI (1970) | uterus |
| Group I (Al-Aubaidi) | B 142 P | Aarhus Univ. | FABRICANT (1950) | lung |
| Group K (Al-Aubaidi) | B 107 PA | Aarhus Univ. | AL-AUBAIDI (1970) | ? |
| Group L(Al-Aubaidi) | B 144 P | Aarhus Univ. | MOULTON (1956) | joint |
| *A. modicum* | PG 49 (Squire) | Aarhus Univ. | LANGER and CARMICHAEL (1963) | lung |
| *M. agalactiae* subsp. *agalactiae* | PG 2 | Aarhus Univ. | LOPEZ (1934) | udder (sheep) |

*Growth inhibition test.* The GIT was performed according to the technique described by STANBRIDGE and HAYFLICK (1967). Agar plates were prepared in glass Petri dishes (Orion, Hungary) with a diameter of 6 cm, containing 9 ml of agar medium B. The plates were inoculated by "running drop technique" with 0.01 ml of concentrated and diluted to $10^{-1}$, $10^{-2}$ and $10^{-3}$ test cultures. After drying of the inoculated plates at room temperature, filter paper discs (6.0 mm in diameter) soaked with 0.02 ml of antiserum were placed in the middle of the mycoplasma streak. Plates were incubated at 37 °C in wet chambers for 4 days. Simultaneously colony forming units (CFU) of each test culture were determined by plating to find out the proper dilution containing $10^5$ CFU. Results shown in Table II are data of diameters of growth inhibition zones obtained with the dilution of culture containing $10^5$ CFU.

*Metabolic inhibition test.* The tested strains were grown in 30 ml medium B containing 2.0 ml of PPLO Serum Fraction (Difco) instead of horse serum. The medium incubated to the end of log. phase was frozen in 1.0 ml aliquots at —20 °C. For the tests media BG, BA and BT described by ERNØ *et al.* (1973) were used. Medium BG was used for testing *M. mycoides* subsp. *mycoides* (PG1), *M. bovirhinis* (PG43), *A. laidlawii A* (PG8), *A. laidlawii B* (PG9), *A. modicum* (PG49 Squire), *A. axanthum* (ATCC 25176), serogroups 7 (PG50), K (B 107PA) and L (B 144P). Because of weak TTC-reduction by *M. bovigenitalium* (PG11) and *M. bovigenitalium* (Kalle HE1) for the metabolic inhibition test the later strain was used in medium BG. Decrease of pH in this case was approximately 0.4 units only. The following strains were tested in medium BA: *M. arginini* (G230), *M. alkalescens* (PG51), serogroups H (B 139P) and I (B 142P), *M. gateae* (CS), *M. gallinarum* (PG16). *M. agalactiae* subsp. *agalactiae* (PG2) and *M. agalactiae* subsp. *bovis* (Donetta) were tested in medium BT. Titration of antigens and serum samples was performed according to the description of ERNØ *et al.* (1973).

## Results

*Growth inhibition.* The results are demonstrated in Table II. Satisfactory reactions were produced with both sera prepared by us in the homologous systems like with the reference sera. A distinct zone of at least 1.5 mm or more was demonstrated with most of the strains. Partial inhibition was observed with sera against *A. laidlawii A* (PG8) only.

Two-way cross-reactions of the same intensity were found between *A. laidlawii A* (PG8) and *A. laidlawii B* (PG9) strains. One-way cross-reactions were observed between serogroup 7 (PG50) and group L (B 144P) but inhibition zones were weaker than in the homologous system. The strains B 139 P, B142 P and B107 PA of Al-Aubaidi's groups H, I and K were significantly inhibited by *M. gateae* (CS), *M. gallinarum* (PG16) and *A. axanthum*

**Table II**

Comparison of bovine Mycoplasmas and Acholeplasmas in growth inhibition test

| Antisera | | Type or reference strains | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | PG 11 | PG 43 | G 230 | PG 8 | PG 9 | PG 1 | Don. | PG 50 | PG 51 | B 139 P | B 142 P | B 107 PA | B 144 P | PG 49 | PG 2 |
| PG 11 | T | 6.0 | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| 558 | O | 4.5 | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| 559 | O | 5.7 | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| PG 43 | T | — | 6.0 | — | — | — | — | — | — | — | — | — | — | — | — | — |
| 536 | O | — | 5.0 | — | — | — | — | — | — | — | — | — | — | — | — | — |
| 537 | O | — | 6.0 | — | — | — | — | — | — | — | — | — | — | — | — | — |
| G 230 | T | — | — | 2.4 | — | — | — | — | — | — | — | — | — | — | — | — |
| 552 | O | — | — | 6.5 | — | — | — | — | — | — | — | — | — | — | — | — |
| 553 | O | — | — | 2.7 | — | — | — | — | — | — | — | — | — | — | — | — |
| PG 8 | T | — | — | — | 2.0p | 3.0p | — | — | — | — | — | — | — | — | — | — |
| 2807 | O | — | — | — | 2.0 | 2.0p | — | — | — | — | — | — | — | — | — | — |
| 2808 | O | — | — | — | 1.0 | 1.5p | — | — | — | — | — | — | — | — | — | |
| PG 9 | T | — | — | — | 1.5 | 4.0p | — | — | — | — | — | — | — | — | — | — |
| 592 | O | — | — | — | 3.3 | 4.1 | — | — | — | — | — | — | — | — | — | — |
| 593 | O | — | — | — | 3.5 | 6.4 | — | — | — | — | — | — | — | — | — | — |
| PG 1 | E | — | — | — | — | — | 4.2 | — | — | — | — | — | — | — | — | — |
| 568 | O | — | — | — | — | — | 2.0 | — | — | — | — | — | — | — | — | — |
| 569 | O | — | — | — | — | — | 5.0 | — | — | — | — | — | — | — | — | — |

| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Don. | E | — | — | — | — | — | — | 6.5 | — | — | — | — | — | — | — | — |
| 550 | O | — | — | — | — | — | — | 5.5 | — | — | — | — | — | — | — | — |
| 551 | O | — | — | — | — | — | — | 6.0 | — | — | — | — | — | — | — | — |
| PG 50 | E | — | — | — | — | — | — | — | 3.0 | — | — | — | — | 2.6 | — | — |
| 538 | O | — | — | — | — | — | — | — | 3.1 | — | — | — | — | 4.0 | — | — |
| 539 | O | — | — | — | — | — | — | — | 3.9 | — | — | — | — | 1.0 | — | — |
| PG 51 | E | — | — | — | — | — | — | — | — | 4.9 | — | — | — | — | — | — |
| 583 | O | — | — | — | — | — | — | — | — | 2.6 | — | — | — | — | — | — |
| 584 | O | — | — | — | — | — | — | — | — | 3.9 | — | — | — | — | — | — |
| 562 | O | — | — | — | — | — | — | — | — | — | 5.0 | — | — | — | — | — |
| 563 | O | — | — | — | — | — | — | — | — | — | 3.3 | — | — | — | — | — |
| 554 | O | — | — | — | — | — | — | — | — | — | — | 5.0 | — | — | — | — |
| 555 | O | — | — | — | — | — | — | — | — | — | — | 5.0 | — | — | — | — |
| 617 | O | — | — | — | — | — | — | — | — | — | — | — | 2.8 | — | — | — |
| 619 | O | — | — | — | — | — | — | — | — | — | — | — | 3.0 | — | — | — |
| 572 | O | — | — | — | — | — | — | — | — | — | — | — | — | 3.5 | — | — |
| 573 | O | — | — | — | — | — | — | — | — | — | — | — | — | 4.2 | — | — |
| PG 49 | L | — | — | — | — | — | — | — | — | — | — | — | — | — | 2.5 | — |
| 757 | O | — | — | — | — | — | — | — | — | — | — | — | — | — | 3.5 | — |
| 767 | O | — | — | — | — | — | — | — | — | — | — | — | — | — | 5.0 | — |
| PG 2 | E | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 5.7 |
| 548 | O | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 1.5 |

*Notes:* T, TULLY; E, ERNØ; L, LEACH; O, Own; p, partial inhibition

**Table III**

Comparison of bovine Mycoplasmas and Acholeplasmas in metabolic inhibition test

| Antisera | | Type or reference strains | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Ka 11 e HE 11 | PG 43 | G 230 | PG 8 | PG 9 | PG 1 | Don. | PG 50 | PG 51 | B 139 P | B 142 P | B 107 PA | B 144 P | PG 49 | PG 2 |
| PG 11 | T | 5120 | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| 558 | O | 1280 | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| 559 | O | 10240 | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| PG 43 | T | — | 5120 | — | — | — | — | — | — | — | — | — | — | — | — | — |
| 536 | O | — | 2560 | — | — | — | — | — | — | — | — | — | — | — | — | — |
| 537 | O | — | 1280 | — | — | — | — | — | — | — | — | — | — | — | — | — |
| G 230 | T | — | — | 320 | — | — | — | — | — | — | — | — | — | — | — | — |
| 552 | O | — | — | 5120 | — | — | — | — | — | — | — | — | — | — | — | — |
| 553 | O | — | — | 320 | — | — | — | — | — | — | — | — | — | — | — | — |
| PG 8 | T | — | — | — | 2560 | 10240 | — | — | — | — | — | — | — | — | — | — |
| 2807 | O | — | — | — | 640 | 1280 | — | — | — | — | — | — | — | — | 10 | — |
| 2808 | O | — | — | — | 1280 | 640 | — | — | — | — | — | — | — | — | 10 | — |
| PG 9 | T | — | — | — | 2560 | 2560 | — | — | — | — | — | — | — | — | — | — |
| 592 | O | — | — | — | 320 | 640 | — | — | — | — | — | — | — | — | 10 | — |
| 593 | O | — | — | — | 1280 | 1280 | — | — | — | — | — | — | — | — | 10 | — |
| PG 1 | E | — | — | — | — | — | 5120 | — | — | — | — | — | — | — | — | — |
| 568 | O | — | — | — | — | — | 5120 | — | — | — | — | — | — | — | — | — |
| 569 | O | — | — | — | — | — | 5120 | — | — | — | — | — | — | — | — | — |

| | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Don. | E | — | — | — | — | — | — | 10240 | — | — | — | — | — | — | — | 80 |
| 550 | O | — | — | — | — | — | — | 10240 | — | — | — | — | — | — | — | 20 |
| 551 | O | — | — | — | — | — | — | 5120 | — | — | — | — | — | — | — | 80 |
| PG 50 | E | — | — | — | — | — | — | — | 1280 | — | — | — | — | 320 | — | — |
| 538 | O | — | — | — | — | — | — | — | 2560 | — | — | — | — | 20 | — | — |
| 539 | O | — | — | — | — | — | — | — | 2560 | — | — | — | — | 80 | — | — |
| PG 51 | E | — | — | — | — | — | — | — | — | 10240 | — | — | — | — | — | — |
| 583 | O | — | — | — | — | — | — | — | — | 5120 | — | — | — | — | — | — |
| 584 | O | — | — | — | — | — | — | — | — | 2560 | — | — | — | — | — | — |
| 562 | O | — | — | — | — | — | — | — | — | — | 1280 | — | — | — | — | — |
| 563 | O | — | — | — | — | — | — | — | — | — | 640 | — | — | — | — | — |
| 554 | O | — | — | — | — | — | — | — | — | — | — | 5120 | — | — | — | — |
| 555 | O | — | — | — | — | — | — | — | — | — | — | 5120 | — | — | — | — |
| 617 | O | — | — | — | — | — | — | — | — | — | — | — | 5120 | — | — | — |
| 619 | O | — | — | — | — | — | — | — | — | — | — | — | 2560 | — | — | — |
| 572 | O | — | — | — | — | — | — | — | 40 | — | — | — | — | 10240 | — | — |
| 573 | O | — | — | — | — | — | — | — | 80 | — | — | — | — | 5120 | — | — |
| PG 49 | L | — | — | — | — | — | — | — | — | — | — | — | — | — | 1280 | — |
| 757 | O | — | — | — | — | — | — | — | — | — | — | — | — | — | 1280 | — |
| 767 | O | — | — | — | — | — | — | — | — | — | — | — | — | — | 5120 | — |
| PG 2 | E | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 10240 |
| 548 | O | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 10240 |

*Notes:* T, TULLY; E, ERNØ; L, LEACH; O, Own

(ATCC 25176) sera respectively. The sera produced against Al-Aubaidi's serogroups gave an inhibition zone with the mentioned species nearly as much as their homologous sera (Table III).

*Metabolic inhibition.* Sufficient homologous reactions were observed with newly prepared and with the reference sera too (Table III). The homologous titers varied in a range of 1 : 320 to 1 : 10240. Reactions with serum dilution 1 : 10 and more were regarded as positive. Two-way cross-reactions of the same strength were demonstrated between *A. laidlawii* A (PG8) and *A. laidlawii* B (PG9) strains. Two-way cross-reaction was also obtained between serogroups 7 (PG50) and L (B 144P) but the heterologous reactions were weaker than that of the homologous system. Metabolic activity of *M. agalactiae* subsp. *agalactiae* (PG2) was inhibited by *M. agalactiae* subsp. *bovis* (Donetta) serum. The titres of these reactions were also weaker than in the homologous system. Two-way cross-reaction was demonstrated between serogroup H (B 139P) and *M. gateae* (CS), serogroup I (B 142P) and *M. gallinarum* (PG16), serogroup K (B 107PA) and *A. axanthum* (ATCC 25176) respectively (Table IV).

## Discussion

The present studies confirm most of the data published by Ernø *et al.* (1973) and Ernø and Jurmanová (1973). Most of the mycoplasmas and acholeplasmas concerned are serologically different from each other. However some of the strains showed cross-reactions. Our findings are in conformity with the observations of Ernø *et al.* (1973) concerning inhibition of sero-group 7 (PG50) by the sera against serogroup L (B 144P) in MIT but we didn't observe any inhibition of group L (B144 P) with *M. mycoides* subsp. *mycoides* (PG1) and *M. bovigenitalium* (PG11) in MIT and inhibition of serogroup 7 (PG50) with *M. bovigenitalium* (PG11) and *M. arginini* (G230) sera in MIT and *M. mycoides* subsp. *mycoides* (PG1), *M. bovigenitalium* (PG11) as well as serogroup L (B 144P) sera in GIT.

Since the serological examination of *M. mycoides* subsp. *mycoides* (PG1), group 7 (PG50) and group L (B 144P), sharing major biochemical characteristics (Ernø and Stipkovits, 1973) showed some relationship in Aarhus and in our laboratory it is wise to follow the suggestion proposed by Ernø *et al.* (1973) not to attempt any formal classification of these strains till more informations, including DNA homologies will be obtained.

On the basis of the results obtained there is no reason to suppose any antigen relationship between strains of distinct biochemical characters like group 7 (PG50), group L/B (B 144P) and *M. bovigenitalium* (PG11) as well as *M. arginini* (G230) suggested by Cottew (1970).

Our findings are corresponding with Leach's data (1973) concerning the results of examination of *A. modicum* (PG49 Squire). This strain was in-

**Table IV**

Identification of serogroups H, I and K by means of the MIT and GIT

| Species or serogroups | Tests | Hyperimmune sera | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Group H(B 139 P) | | *M. gateae* CS Erno | Group I(B 142 P) | | *M. gallinarum* PG 16 Tully | Group K(B 107 PA) | | *A. axanthum* | |
| | | 562 | 563 | | 554 | 555 | | 617 | 619 | ATCC 570 | 25176 571 |
| | | Own | | | Own | | | Own | | Own | |
| Group H | GIT | 5.0 | 3.3 | 3.5 | — | — | — | — | — | — | — |
| (B 139 P) | MIT | 1280 | 640 | 10240 | — | — | — | — | — | — | — |
| *M. gateae* | GIT | 2.5 | 2.2 | 5.0 | — | — | — | — | — | — | — |
| (CS) | MIT | 2560 | 5120 | 10240 | — | — | — | — | — | — | — |
| Group I | GIT | — | — | — | 5.0 | 5.0 | 6.5 | — | — | — | — |
| (B 142 P) | MIT | — | — | — | 5120 | 5120 | 10240 | — | — | — | — |
| *M. gallinarum* | GIT | — | — | — | 2.5 | 2.3 | 6.5 | — | — | — | — |
| (PG 16) | MIT | — | — | — | 160 | 80 | 2560 | — | — | — | — |
| Group K | GIT | — | — | — | — | — | — | 2.8 | 3.0 | 3.0 | 4.0 |
| (B 107 PA) | MIT | — | — | — | — | — | — | 5120 | 2560 | 2560 | 1280 |
| *A. axanthum* | GIT | — | — | — | — | — | — | 3.0 | 2.8 | 3.0 | 1.8 |
| (ATCC 25176) | MIT | — | — | — | — | — | — | 160 | 160 | 2560 | 640 |

hibited by the homologous sera only, inhibitions by all other sera in low dilutions (1 : 10) are to be considered as non-specific reactions. Our data confirmed Leach's suggestion to accept this group as a new species.

It was also confirmed that one-way cross-reaction exists between *M. agalactiae* subsp. *agalactiae* (PG2) antigen and *M. agalactiae* subsp. *bovis* (Donetta) antisera (ERNØ *et al.* 1973).

There was not any reaction between *M. alkalescens* and other species or serogroups supporting LEACH's suggestion (1973) to accept it as a new species.

Our results of cross-testing serogroups H, I and K (AL-AUBAIDI and FABRICANT, 1971) with the reference sera of *M. gateae*, *M. gallinarum* and *A. axanthum* respectively are in conformity with the observations of ERNØ *et al.* (1973) too.

On the basis of these findings serogroup H belongs to *M. gateae*, group I to *M. gallinarum* and serogroup K to *A. axanthum* species. Since the first two species had been isolated from other animal sources (not bovine) the term of "bovine mycoplasma" for these species should be used with caution. *A. axanthum* has been known as a tissue culture contaminant (TULLY and RAZIN, 1970) in light of our results the bovine origin of *A. axanthum* could be accepted since three strains had been isolated from the nasal cavity and lung of cattle suffering from catarrhal pneumonia being *A. axanthum*.

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Thanks are due to Miss M. HANGODI for excellent technical assistance.

## Summary

*Mycoplasma* and *Acholeplasma* reference strains of bovine origin were tested in growth inhibition and metabolic inhibition tests. Two-way cross-reaction was demonstrated between serogroup 7 PG50 (Leach) and serogroup L B 144P(Al-Aubaidi) in MIT and one-way cross-reaction in GIT. Metabolic activity of *M. agalactiae* subsp. *agalactiae* (PG2) was inhibited by *M. agalactiae* subsp. *bovis* (Donetta) antisera.

Serogroups designated H, I and K (Al-Aubaidi) showed significant two-way cross-reactions in both tests with *M. gateae*, *M. gallinarum* and *A. axanthum* species confirming observations of ERNØ, JURMANOVÁ and LEACH (1973).

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Address of the authors: Dr. László STIPKOVITS, Lea VARGA, 1581 Budapest, Hungária krt. 21, Hungary.

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# THE FINE STRUCTURE OF *LANKESTERELLA* SP. SPOROZOITES PARASITIC IN THE FROG *RANA PIPIENS*.* **

By

G. HELLER

Zoological Institute, University of Bonn, Germany
and University of Illinois, College of Veterinary Medicine, Urbana

(Received August 22, 1973)

Studies on the blood-inhabiting protozoa of the frog *Rana pipiens* revealed two different species of parasites (HELLER, 1972a): a haemogregarine and a small, sporozoite-like form similar to *Lankesterella* sporozoites described from frogs and toads (NÖLLER, 1913, 1920; LABBÉ, 1894; FANTHAM *et al.*, 1942; STEHBENS, 1966; MANSOUR and HELMY MOHAMMED, 1962). The latter form occurs both within erythrocytes and also within endothelial cells of the liver, lung and spleen. Since its life cycle is unknown, electron microscope studies have been made in order to compare its fine structure with those of other *Lankesterella* species described by earlier authors (GARNHAM *et al.*, 1962; STEHBENS, 1966; BÜTTNER, 1968).

## Material and Methods

Small pieces of the liver, lung and spleen of *Rana pipiens*** infected with *Lankesterella* sp. were fixed in 3% glutaraldehyde adjusted to pH 7.2 with phosphate buffer, and postfixed in 1% osmium tetroxide. The fixed material was washed several times in phosphate buffer, dehydrated in a graded series of ethanol followed by propylene oxide, and embedded in Epon. Sections, made with a Reichert ultratome, were stained with uranylacetate and lead citrate, and observed with a Hitachi HU 11C electron microscope.

## Results

The fine structure of the erythrocyte-inhabiting forms is identical to that of the forms in endothelial cells (Figs 1, 2, 3, 5). They have all the typical organelles of a coccidian sporozoite, including a three-layered pellicle (Fig. 7), about 30 subpellicular microtubules (Fig. 2), an apical complex with a conoid, two preconoidal rings, a polar ring and a rhoptry-microneme complex (Figs

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** Some of these results were presented at the 25th annual meeting of the Society of Protozoologists at Minneapolis, Minn., Aug. 28—Sept. 1, 1972.

*** The frogs were delivered by Nasco, E. G., Steinhilber & Co., Inc., Fort Atkinson, Wisconsin 53538, USA.

1, 3). Micropores are visible (Fig. 3). Furthermore there are two refractile bodies (Fig. 3) which are the most characteristic organelles of sporozoites. The Golgi apparatus is anterior to the nucleus (Fig. 3).

Sausage-like organelles with inner, parallel membranes which are perpendicular to the organelles' longitudinal axis are present (Figs 4, 5).

Close to the surface there are vacuoles with membranous and amorphous contents (Fig. 3). Some specimens contain several amylopectin granules (Figs 2, 5, 6).

Furthermore the shape of the pellicle seems to be quite interesting. There are infoldings (Figs 3, 6) at the posterior part of the body which contain tubular or vesicle-like structures.

In some specimens the pellicle is folded only slightly (Figs 1, 3, 6); these "waves" seem to be more or less regular. The subpellicular microtubules (Fig. 3) are straight and do not follow the evagination of the pellicle.

At the anterior end there are slight waves, each one correponding to a subpellicular microtubule (Fig. 3).

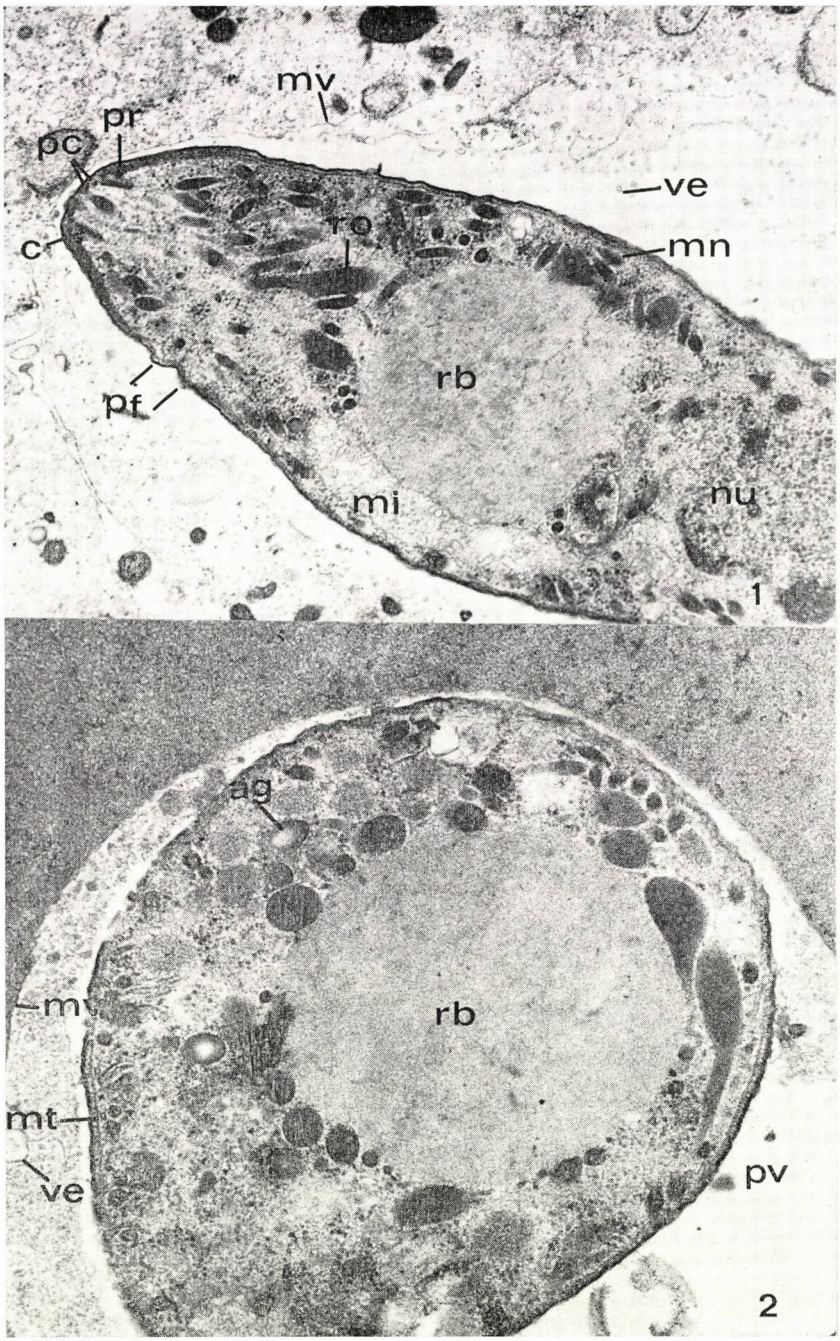
The parasite lies within a parasitophorous vacuole which is membrane-bound (Figs 1, 2, 5), and sometimes filled with small vesicles or tubules (Figs 1, 2, 3, 5, 7). Some of these vesicles are pinched off from the host cell membrane (Fig. 7).

The infected cells show no other evidence of damage; the surface of the infected erythrocytes is smooth and there are only a few small vesicles or cisternae within their cytoplasm (Fig. 3).

## Discussion

The fine structure of *Lankesterella* sp. sporozoites from *Rana pipiens* as revealed by transmission electron microscopy is similar to that of other coccidian sporozoites (PORCHET-HENNERÉ and VIVIER, 1971; RYLEY, 1969; BARDELE, 1966; ROBERTS *et al.*, 1970a, 1970b, 1971) and almost identical with that of sporozoites of other *Lankesterella* species (GARNHAM *et al.*, 1962; STEHBENS, 1966; BÜTTNER, 1968). The sausage-like organelles with the inner membranes, also described from *L. garnhami* and *L. hylae*, are especially interesting. As far as I know such organelles have not been described from any other sporozoites. These organelles may therefore be characteristic of

*Figs 1—2. Lankesterella* sp. *1*, sporozoite within an endothelial cell of the liver, 24000×; *2*, cross-section of an intraerythrocytic sporozoite, 32000×. Abbreviations for all figures: ag, amylopectin granule; aw, anterior "waves"; c, conoid; go, Golgi apparatus ; hn, host cell nucleus; im, inner membranes; mb, membranous bodies; mi, mitochondrium; mn, micronеme; mp, micropore; mt, microtubule; mv, membrane of the parasitophorous vacuole; nu, nucleus; om, outer membrane; pc, preconoidal rings; pf, pellicular folds ("waves"); pi, pellicular infoldings; pr, polar ring; pv, parasitophorous vacuole; rb, refractile body; ro, rhoptry; va, vacuole; ve, vesicle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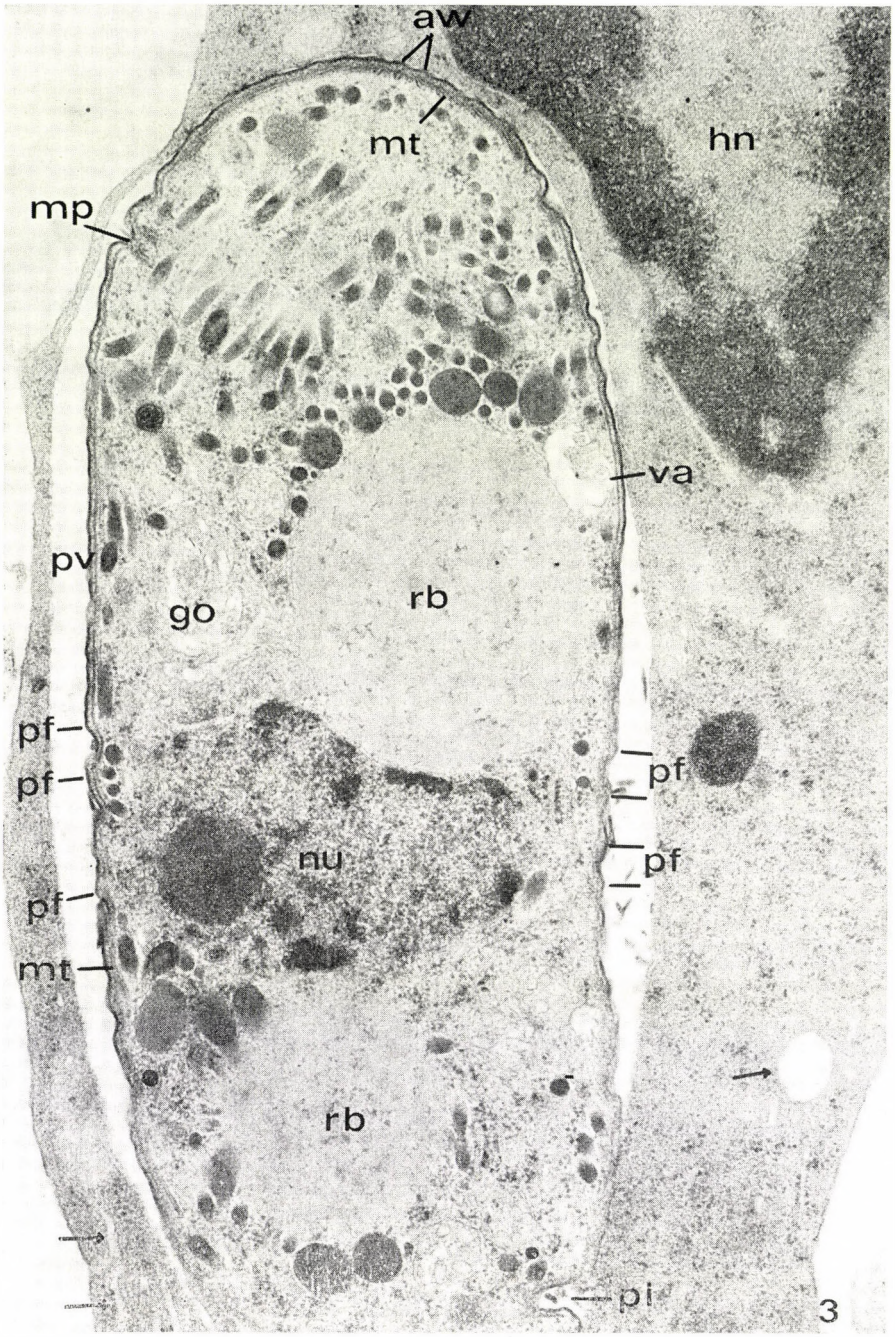
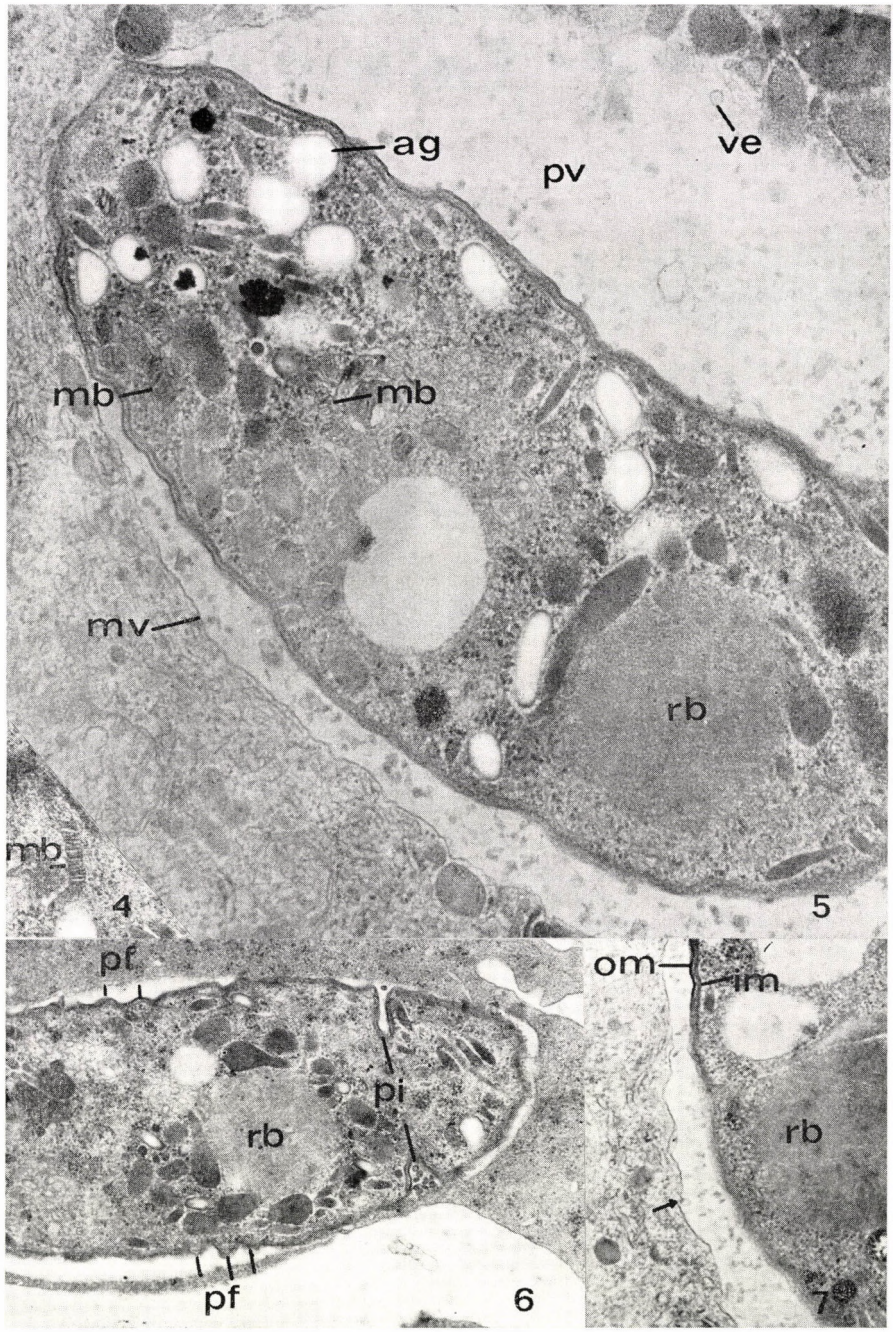



*Fig. 3. Lankesterella* sp. Intraerythrocytic sporozoite: (→) indicates cysternae and vesicles within the host cell cytoplasma, 27000×




*Figs 4—7. Lankesterella* sp. *4*, Membranous body, with inner, parallel membranes (sausage-like organelle), 31500×; *5*, Intraendothelial sporozoite, 32000×; *6*, Intraerythrocytic sporozoite, 18000×; *7*, Intraendothelial sporozoite; (→) indicates a vesicle which is pinched off from the membrane of the parasitophorous vacuole, 25000×

*Lankesterella*. BÜTTNER (1968) suggested that there is a close connection between food vacuoles and the membranous bodies. No such connections have been found in this species from *Rana pipiens*. The finding of those organelles, the typical shape of the parasites, and their location within erythrocytes and endothelial cells let us suppose that the organism is a *Lankesterella* species.

Furthermore the amount of amylopectin within the parasites leads to the belief that they have been in their host cells for a considerable length of time without development. The sporozoites of *Lankesterella* must enter a second host, a "transmitter", before they are able to infect another specific vertebrate host (NÖLLER, 1913, 1920); LAINSON, 1959).

The observations on the parasitophorous vacuole are similar to those of the earlier authors.

The "waves" of the pellicle are similar to those described by ROBERTS *et al.* (1971) and DESSER (1970). The fact that there are only a few such waves on an almost longitudinal section may be explained as due to a pimple-like structure (HELLER, 1972b). The slight waves at the anterior part are identical with the anterior ridges which correspond to the subpellicular microtubules (HELLER, 1972 b). These waves seem to explain the movement of the organisms. The *Lankesterella* sporozoites were apparently fixed during movement within the parasitophorous vacuole. Thuis suggestion is supported by light microscope observations on living *Lankesterella* sporozoites which leave the erythrocytes almost immediately after blood has been taken from the animal.

The infoldings of the pellicle at the posterior end are similar to those described by BÜTTNER (1968; Fig. 5a), but the channel-like infolding that the described (Fig. 5b) was not found. In contrast these infoldings result from bending of the posterior end of the sporozoite, as seen in serial sections and observed with the light microscope.

## Acknowledgement

I should like to thank Dr. Norman D. LEVINE for his advice and assistance in the preparation of this paper.

## Summary

The fine structure of *Lankesterella* sp. sporozoites is almost identical with that of other *Lankesterella* species; membraneous organelles (sausage-like organelles) seem to be especially characteristic of all these species. The pellicle is slightly folded during movement. The shape of these folds or "waves" is compared with that of *Eimeria stiedai* sporozoites as revealed with the scanning electron microscope (HELLER, 1972b). The parasitophorous vacuole is filled with small vesicles which are pinched off from the host cell membrane.

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Address of the author; Dr. Gert HELLER, Bonn, Hundgasse 9 DBR

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# IMMUNOLOGICAL STUDY OF SWINE TRANSMISSIBLE GASTROENTERITIS (TGE)

By

J. BENYEDA, E. MOCSÁRI and GY. GAJDÁCS

Department of Epizootiology (Head: J. MÉSZÁROS), University of Veterinary Science, Central Veterinary Institute (Director: A. VÁNYI), Budapest, and Veterinary Institute (Director: O. PROKOPOVITSCH) Békéscsaba

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The usual mechanism of passive protection by colostral antibodies includes entering of antibodies into the circulating blood of the newborn animal. In connection with several infectious diseases, however, including the virus diarrhoea (transmissible gastroenteritis, TGE) of swine, the mechanism of passive immunity is different.

Several authors (ÁLDÁSY *et al.*, 1966; CSONTOS and SZENT-IVÁNYI, 1971; MÉSZÁROS *et al.*, 1967; SZENT-IVÁNYI *et al.*, 1964) observed in epizootological studies of TGE that piglets born 2—3 weeks after the recovery of the sow did not develop the disease, although the agent was stil present in the herd. It was shown later that in such cases protection was due to colostral antibodies (BOHL *et al.*, 1972; CSONTOS *et al.*, 1973), but not *via* the blood stream; in the case of TGE, continuous intake of colostral and milk antibodies is required to neutralize ingested virus in the intestinal tract, before these could penetrate the intestinal epithelial cells of the piglet (BOHL *et al.*, 1972; CSONTOS *et al.*, 1973; HOOPER and HAELTERMAN, 1966).

Information was lacking on the antibody level required to protect piglets born in an infected environment and on the relation of milk and colostral antibody titres to the ceasing of the epizooty. Studies on a field outbreak of TGE are reported in this paper to throw some light on these problems.

## *Description of the outbreak*

The outbreak occurred in a traditionally managed swine farm, where the sow herd consisted of 500 animals.

Onset of the disease was first observed among feeder swine on 22 January 1972. The sow herd became involved on 28—30 January and the symptoms subsided after 6 days. Piglets up to 7 days old, born before the onset of the epizooty, all became diseased and died within 2—4 days. The same occurred to the piglets born within 14 days from the onset. From the 14th day on survivors were found in several diseased litters. Among the piglets born 15—17 days after the outbreak losses varied between 50 and 100% in most of the litters, but in a few litters, piglets did not become involved at all.

Neither disease, nor losses occurred among the piglets born after the 17th day. Losses were recorded for 7 days.

During the period of epizootological observation, blood and milk samples were collected from 29 sows at different intervals for virus neutralization test.

### *Collection of test material*

Samples of blood and milk were collected from the sows between 12 and 24 days after the onset of the epizooty. As the outbreak occurred in a farrowing period, piglets were born nearly every day. Milk samples were withdrawn on the day of farrowing and 3, 5 and 7 days later at random from 1—2 teats. Blood samples were taken on the day of farrowing and 7 days later from the cranial vena cava. Fifteen I.U. of oxytocin were injected into the cranial vena cava prior to milk sampling; milk secretion began 1—3 minutes after the injection.

### *Virus strain*

The CKp strain (Csontos *et al.*, 1973) was used for virus neutralization test.

### *Blood sera and colostral (milk) whey*

Milk samples were skimmed by centrifugation, then 1/10th volume of a 10% solution of clotting substance was added and the sample was incubated for 60 minutes at 37 °C, after a second centrifugation the whey was collected by pipetting. Serum and whey samples were inactivated at 56 °C for 30 minutes and stored at —20 °C until tested.

### *Virus neutralization test*

Serial dilutions of serum were added to 100 $TCID_{50}$/0.1 ml virus. The virus-serum mixture was incubated for 60 minutes at 37 °C and 0.2 ml amounts from each dilution were inoculated into 2—3 tubes of secondary swine thyroid cell culture. Final reading was made 3 days after inoculation. The antibody titres of serum and whey were expressed as the highest dilution producing complete inhibition of viral cytopathic effect.

## Results

Virus-neutralizing antibodies began to appear in serum and whey samples from sows 14 days after the onset of illness. All the sows farrowed between the 15th—17th day after the onset of the epizooty had antibodies,

although some of them at scarcely measurable levels. The titres rose gradually until the closing of observation on the 21st to 23rd day.

Mean antibody titres were calculated from four milk samples and two serum samples from each sow. Group averages were then calculated from individual mean values for the periods shown in Fig. 1. The average antibody levels thus obtained were then plotted against piglet losses (Fig. 1).

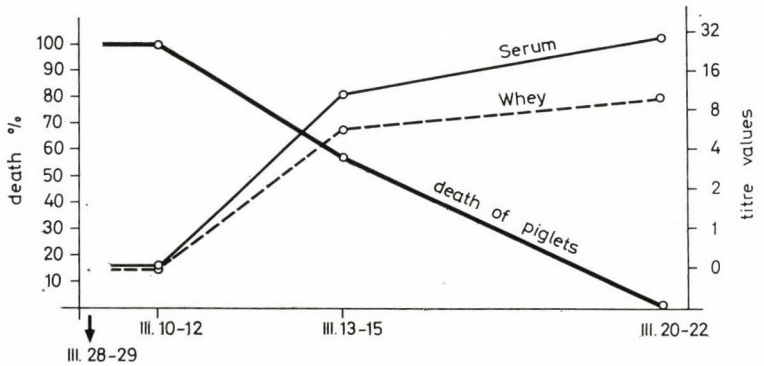


*Fig. 1.* The relationship between the serum and milk antibody levels of sows recovered from TGE and mortality rate of their newborn piglets

Individual serum and whey titres showed considerable variations, ranging from 1 : 1 to 1 : 256 and 1 : 1 to 1 : 64, respectively. Whey antibody levels did not notably change in the majority of the cases between the first and seventh day after farrowing, whereas serum antibody levels were, with the exception of 6 sows, higher after 7 days than on the day of farrowing.

## Discussion

The present studies disclosed a close inverse correlation between milk and serum antibody levels of the sow and the losses from TGE. No antibodies were found in the serum and milk of sows until 14 days after onset. In accordance with this, piglet mortality approximated to 100% during that period.

Those sows which farrowed between 15 and 17 days after onset had various levels of virus-neutralizing antibodies in serum and milk. Accordingly, piglet losses began to decrease also in affected litters.

Some of the litters born in that period were already free from disease. An inverse correlation could be demonstrated between the antibody contents of sow's milk and blood and piglet morbidity and, especially, mortality. The epizooty ceased also in the suckling piglets by the 21st—23rd day, whereafter no further piglet became ill (See Fig. 1).

Disease incidence among piglets did not immediately cease upon the appearance of antibodies in colostrum and milk, owing to the initial low level

of antibodies and to the still heavily infected environment. The present findings suggest that the protection of piglets from TGE requires a mean colostral and milk antibody level of 1 : 8 or higher; this antibody level prevents infection in the majority of the cases and suppresses the intestinal multiplication of the virus in the piglets which yet become infected; thereby spreading of virus to the environment is considerably reduced. Reduction of environmental contamination also seems to play an important role in the ceasing of the outbreak.

In most cases, milk antibody level did not notably change in the first week after farrowing. This seems to contradict the prevailing concept that colostral and milk antibody titres do not change for two days after farrowing, then decrease 2—8 times during the subsequent 2—3 days and remain at that level for a longer period (Csontos *et al.*, 1973). The controversial findings seem to be explainable by the fact that in the present study the immune response of the sows had not reached its peak by the time of farrowing. The observation that all but a few sows had higher serum antibody titres after 7 days than at farrowing weighs in favour of this interpretation.

Important conclusions can also be drawn in respect of active immunization; in an immunized herd, no susceptible individuals must be left; such animals might transfer the infection to the progeny of vaccinated, but not fully immune, sows.

## Acknowledgement

The authors are indebted to Mr. L. Belopotoczky for excellent technical assistance.

## Summary

The correlation between the decrease and ceasing of piglet morbidity and mortality from transmissible gastroenteritis (TGE) on the one hand and the increase of colostral and milk antibody levels of the sows on the other was investigated during a field outbreak of TGE. Blood and milk samples were taken from those sows which farrowed between 12 and 23 days after the onset of the epizooty. Blood samples withdrawn on the day of farrowing and 7 days later, and milk samples collected at farrowing and after 3, 5 and 7 days were tested for virus-neutralizing antibodies. The antibody titres showed an inverse correlation with the piglet morbidity and mortality in the period of the epizooty. It was concluded that a mean colostral and milk antibody titre of 1 : 8 or higher is required to stop disease and losses among the suckling piglets.

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Address of the authors: Dr. János Benyeda, 1581 Budapest, P.O.B. 22; Dr. Endre Mocsáry, 1149 Budapest, Tábornok u. 2; Dr. György Gajdács, 5600 Békéscsaba, Szabolcs u. 2, Hungary.

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# STUDIES ON THE DEVELOPMENT OF *EIMERIA LEPORIS (PROTOZOA: SPOROZOA)* AND ON ITS PATHOGENICITY FOR THE HARE (*LEPUS EUROPAEUS* PALL.)

By

L. PELLÉRDY, M. HÖNICH and L. SUGÁR

Department of Parasitology (Head: Prof. T. KOBULEJ), University of Veterinary Science, Budapest and Wildlife Biology Station (Head: T. FODOR), Budakeszi

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*Eimeria leporis* was the first intestinal hare coccidium described as a valide species on the basis of oocyst morphology and negative infection experiments in rabbits (NIESCHULZ, 1923). In the 'twenties, NIESCHULZ regarded all other intestinal coccidia of the hare *(Lepus europaeus)* identical with *Eimeria perforans*, a parasite of the rabbit *(Oryctolagus cuniculus)*. According to present knowledge, *Lepus* species have about 13 Eimerian parasites which can be differentiated from rabbit intestinal coccidia by oocyst morphology. Their host specificity has been proved by negative cross infection experiments (PELLÉRDY, 1956, 1965).

Although the host specificity of *Eimeria leporis* is now obvious, little is known about its other properties. The endogenous cycle and pathogenicity as well as the symptoms and gross lesions occurring in *Eimeria leporis*-coccidiosis are poorly understood. In order to fill this gap of knowledge, carcases of hares collected in the Wildlife Biology Station were examined regularly in great detail and animals were experimentally infected. The findings are reported in this paper.

## Material and Methods

Examination of the carcases of 227 spontaneously died hares submitted to the Wildlife Biology Station in 1972 and 1973 showed that the death of 14 animals had been due to coccidiosis. The Eimerian species occurring in the carcases were identified and their distribution was determined. The intestines of fresh carcases and of hares killed in an advanced stage of coccidiosis were fixed in formalin immediately after removal and the sections prepared from them were stained with haematoxylin and eosin, and Mallory's stain for further study.

The sequence of endogenous stages could not, of course, be precisely established by this method, but schizonts and gamonts originating from infections at different times could be followed up in serial sections and a close study of the various types of schizonts enabled a tentative assessment of the sequence and number of schizogonic generations.

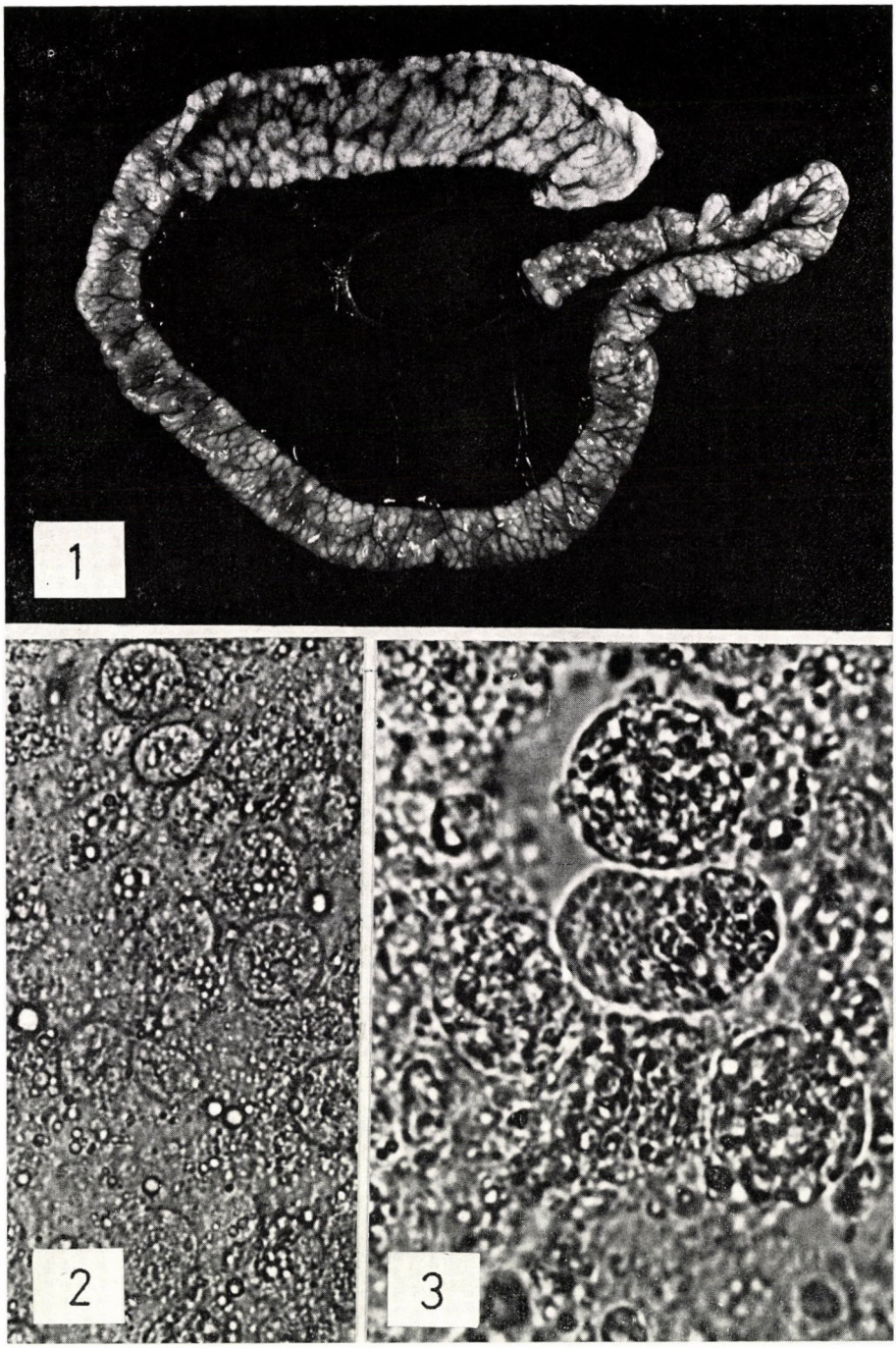
Two hares aged 2.5 to 3 months were available for experimental infection. To determine the prepatent period, these were infected orally with two million sporulated *Eimeria leporis* oocysts each. The infectious material was taken from an about 1-year-old hare, which excreted almost exclusively *Eimeria leporis* oocysts, and was killed in the premortal state. In addition to a redescription of *Eimeria leporis* oocysts, the morphology of the endogenous stages and the prepatent period, pathogenicity of *Eimeria leporis*, and the gross lesions due to the establishment of the coccidia in the intestine, are described and the possible immune response is discussed in this report.

## *Eimeria leporis* Nieschulz, 1923

The oocysts are slim, elliptic or often of a bent bean- or kidney-shape (Figs 4—5). They measure 23—38 by 12—20 $\mu$. These dimensions roughly correspond to these given by NIESCHULZ — 26—38 by 13—20 $\mu$. (average: 32 by 16 $\mu$) — except for the lower limits. The oocyst wall is light, smooth, about 1.5 $\mu$ thick and bilayered; it bears a micropyle which is represented by a 2—4 $\mu$ wide lighter region of the wall at one pole (Figs 4—5). This area is made more conspicuous by glycerol treatment; the oocysts appear to have slightly flattened in the enrichment medium; the micropylar area even becomes indented (Figs 4—5). Oocysts maintained for several days in potassium bichromate solution show creases of the inner layer, converging towards the micropyle (Fig. 5). In freshly passed oocysts, the sporont is elongated or round, 12—14 $\mu$ in diameter, with sharp contours and a granular cytoplasm with well-visible nuclear and nucleolar contours.

Sporulation takes 2—3 days at room temperature. During this period pyramid stages, represented by rectangular or tapering sporoblasts in the oocyst, can be observed. The pyramid stages disappear after half to one hour, suggesting that transformation takes a rapid course. An oocyst residuum appears after sporocyst development, roughly in the centre of the oocyst (Fig. 5); this is usually a regular spherical body with sharp contours, 6—7 $\mu$ in diameter, containing granules of various sizes. The oocysts have no polar bodies.

The sporocysts (Fig. 5) are oval in shape, bluntly tapering at one end, on which they bear a button-like Stieda body. The sporocyst wall is double-contoured. The sporocysts measure 9—10 by 6—7 $\mu$ inside the oocyst, outside they are somewhat larger, 10—14 by 6—8 $\mu$. The sporozoites are narrower at one end compared to the other and a few refractile granules among them seem to represent the sporocyst residuum. Sporozoites released by compression were sausage-shaped and measured 8 by 2 $\mu$.




*Figs 1—3. 1*, partly opened small intestine of hare. Severe intestinal coccidiosis; *2—3*, macrogamonts from mucosal scrapings (unstained). ×500 and 1000

### *Endogenous stages*

Endogenous development could not be studied with the desired accuracy for lack of a pure oocyst material and oocyst-free hares; at all events, these requirements cannot be met with the present experimental technique. Thus our findings are approximations at the probability level of 300—500 : 1, because one in about every 300—500 oocysts found in the faeces of the histologically examined hare belonged to a species other than *Eimeria leporis (Eimeria robertsoni, Eimeria semisculpta, Eimeria europaea* or *Eimeria hungarica).*

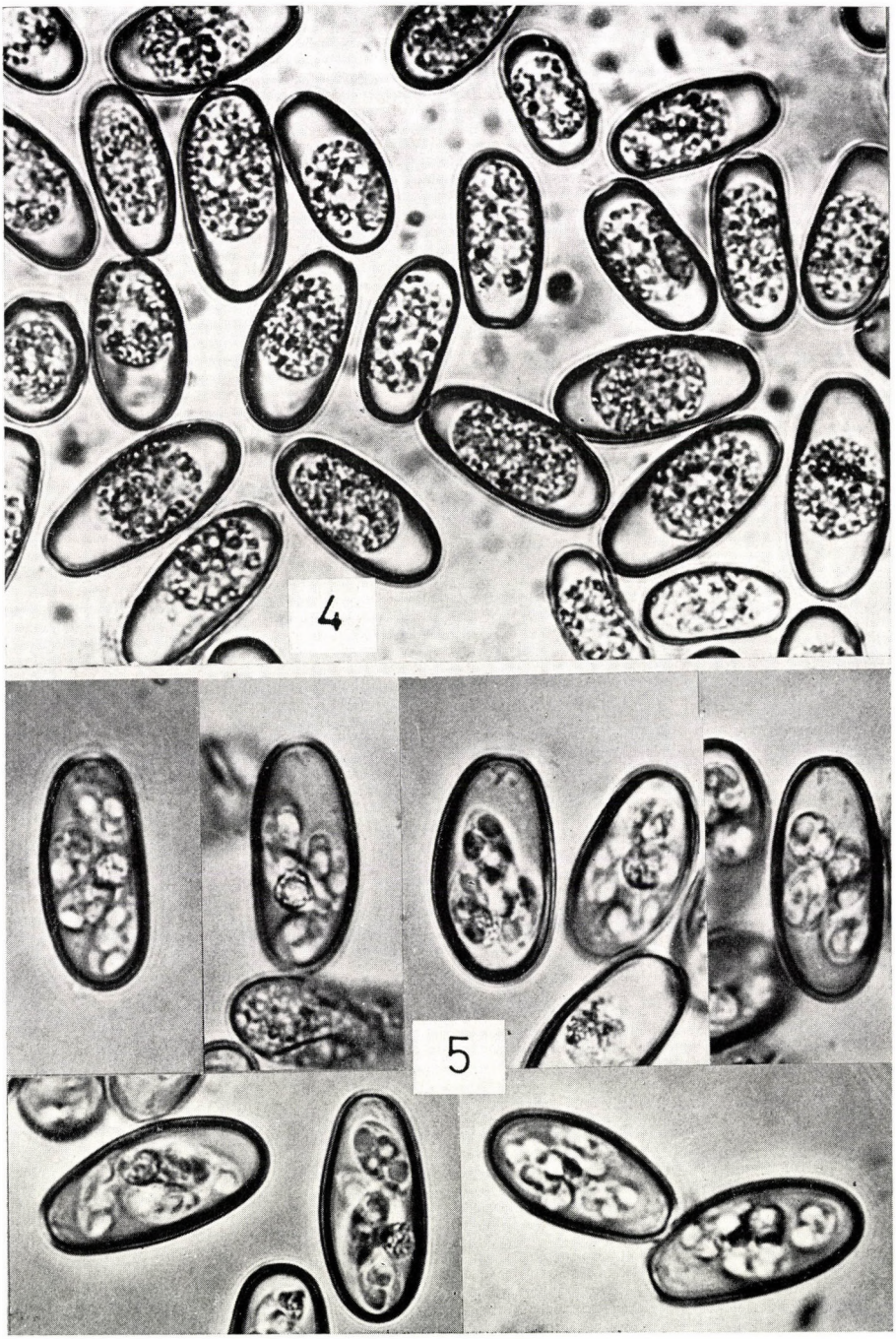
The duration of the prepatent period was determined as one week on the basis of the experimental infection of the two young hares. Faecal samples were examined daily to follow up the time when an abrupt increase of oocyst output took place in the stool of the animals. It should be noted that the two hares had been slightly infected with coccidia by the time of the experimental infection. One rabbit (No. 1) died 4 days after infection and at this time the other animal seemed listless. Hare No. 1 had only few *Eimeria leporis* oocysts in the intestinal contents; No. 2 continued to pass few oocysts, scored as one cross (+) until the 7th day when the quantity of the *Eimeria leporis* abruptly reached the level scored as (++++).

The endogenous stages were studied in sections prepared from the intestines of an about one-year old hare with an almost pure *Eimeria leporis*-coccidiosis, and of hare No. 2, killed in a premortal state 12 days after the experimental infection.

Endogenous stages occurred along the entire length of the small intestine, usually in epithelial cells of the mucous membrane, less often subepithelially, down to the muscularis mucosae (Figs 27—28). Sometimes 3 to 5 parasites were seen in a single epithelial cell (Figs 8, 13, 26, 27 and 28). Endogenous stages of similar age formed islets or foci in the mucous membrane, occasionally forming a coherent coat on one or another intestinal villus (Figs 10, 27 and 28), suggesting that the parasites in each islet belonged to the progeny of merozoites released from a single schizont.

At least two types could be distinguished among the schizogonic stages. The schizonts designated by us as type A (Figs 6, 7, 8) were less often encountered than type B, representing probably an earlier schizogonic generation. This seems the more probable as the majority of the endogenous stages found by us were gamonts (Figs 27 and 28), among which many type-B schizonts representing, in our opinion a later (or probably the last) schizogonic generation, occurred in fairly large numbers.

Type-A schizonts (Figs 6, 7, 8 and 22) are 6—14 $\mu$ in diameter and contain 2—8 (sometimes more) short, stout merozoites, 4.8 by 1.5—3 $\mu$ in size, arranging like the slices of an orange. One or more schizonts are surrounded




*Figs 4—5.* Freshly passed and sporulated *Eimeria leporis* oocysts. ×950 and 1000

by a parasitophorous vacuole resembling a pale halo. The merozoites have centrally or almost centrally placed nuclei and a homogeneous cytoplasm, taking on a dark blue eosin stain. No residuum is present in the schizonts.

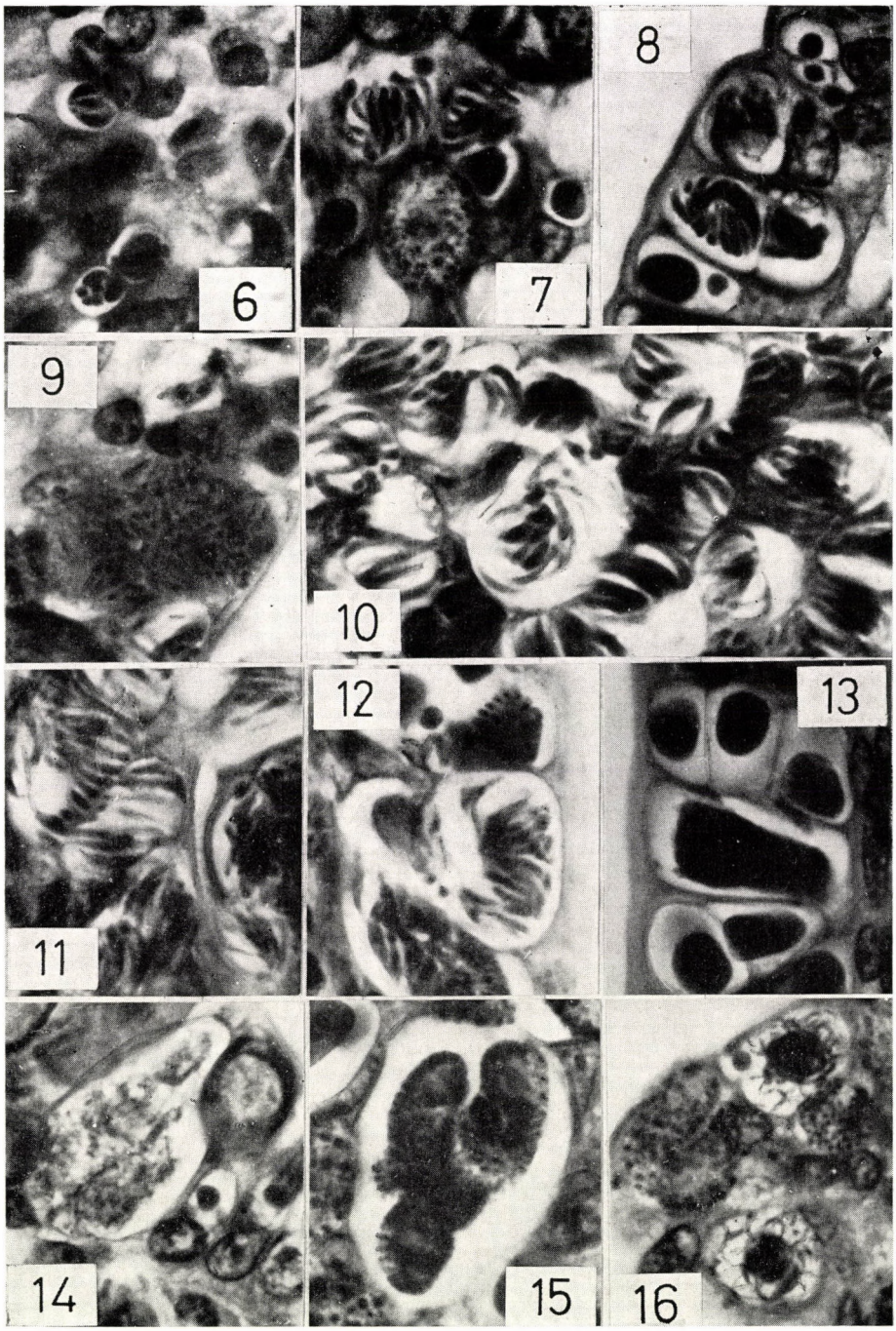
Type B-schizonts (Figs 9, 10, 11 and 12) are 10—20 $\mu$ in diameter, or even larger and have indistinct contours. They are arranging in groups or nests. The smaller — probably younger — forms usually contain 16, the larger — older — ones more (30—50) merozoites. The schizont is surrounded by a distinct parasitophorous vacuole, much larger than the schizont itself. The slim merozoites, 8—10 by 1—1.5 $\mu$ in size, taper at the ends and have the nucleus nearby one pole. The schizonts are arranged in larger groups in the lacteals of the villi; such foci may contain several hundred merozoites. There is no schizont residuum.

A sporadic occurrence of schizonts other than type-A or type-B was also noted. These contained two or four stout, 12—16 $\mu$ long merozoites, in which eight to 10 dark spots, probably corresponding to the nuclei, were seen. Such multinucleated merozoites are known with several Eimeria species and are regarded as the predecessors of a gametogonic generation; there is, however, as yet no evidence to substantiate this assumption.

The macrogamonts (Figs 2, 3, 7, 8, 16, 17 and 20) usually localize subepithelially inside cells. Early macrogamonts (Fig. 28) are 3—5 $\mu$ in diameter and take on a homogeneous haematoxylin stain; often four or five of them may be present in a single cell. A distinctive parasitophorous vacuole appears early around the female gamont. Later contours of nuclei appear in the developing macrogamont; these may reach 5 $\mu$ and inside them the nucleolus is 2 $\mu$ in diameter (Figs 21 and 22). The growing macrogamonts tend to loose staining affinity and blue or eosinophilic granules appear in them. In older female cells the granules recede towards the periphery, thus the entire cytoplasm becomes finely granular and eosinophilic, whereas the nucleus and nucleolus take on a darker haematoxylin stain. In this phase the female gamonts are 20—25 $\mu$ in diameter, the receded granules aggregate along the periphery and fuse into the future oocyst wall. These macrogamonts are already elongated bodies gradually taking the bent elliptic shape of *Eimeria leporis* oocysts (Figs 23, 24 and 25). Oocysts with wall measured 22—32 by 12—18 $\mu$ in histological sections (Fig. 26) and the cytoplasm filled the entire space within the cyst wall.

The microgamonts (Figs 14—19), 3—4 $\mu$ in diameter, have a dark-blue-staining cytoplasm and are surrounded by a parasitophorous vacuole.

*Figs 6—16. 6—8*, Type-A schizonts. Gamonts are also seen in Figs 7 and 8. × about 1000; *9—12*, Type-B schizonts. In Fig. 9, note the young stages, in which no merozoites have yet developed. × about 1000; *13*, Early gametogonic stages. × about 1000; *14—16*, Microgamonts. The beginning of lobulation is well visible in Figs 14 and 16. Two advanced macrogamonts can be seen in Fig. 16. × about 1000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Later the contours of many nuclei appear in the cytoplasm (Figs 15 and 17), the irregularly shaped microgamonts become lobular and attain 15—20 $\mu$ in diameter (Figs 14, 15 and 23). The nuclei arrange along the periphery of the lobules, which later become detached from one another and young microgametes appear in each around a light residuum (Figs 16—19). The microgametes initially resemble splinters, later they elongate to form slim cells, lying parallel to one another and twisting around a centrally placed dark residuum in a regular meridian-like pattern (Fig. 19). The microgametes measure 2.5—4 by 0.3—0.5 $\mu$, the central residuum is about 6—8 $\mu$ in diameter. Mature, in all probability released, microgametes are often found in the relatively large parasitophorous vacuole surrounding the microgamont and between cells in the adjacent tissues. One or more freshly penetrated microgametes are frequently seen in macrogametes.

## *Pathogenicity of* Eimeria leporis

Nieschulz (1923) examining the carcases of 50 hares shot as game, found in those massively infected with coccidia several small greyish-white foci in the small intestines. Such foci if very numerous, greatly interfere with absorption. Nieschulz found chiefly gametogonic stages by histological examination and the relatively small number of oocysts encountered by him in the intestinal lumen clearly showed that pathogenicity had been at peak prior to oocyst development.

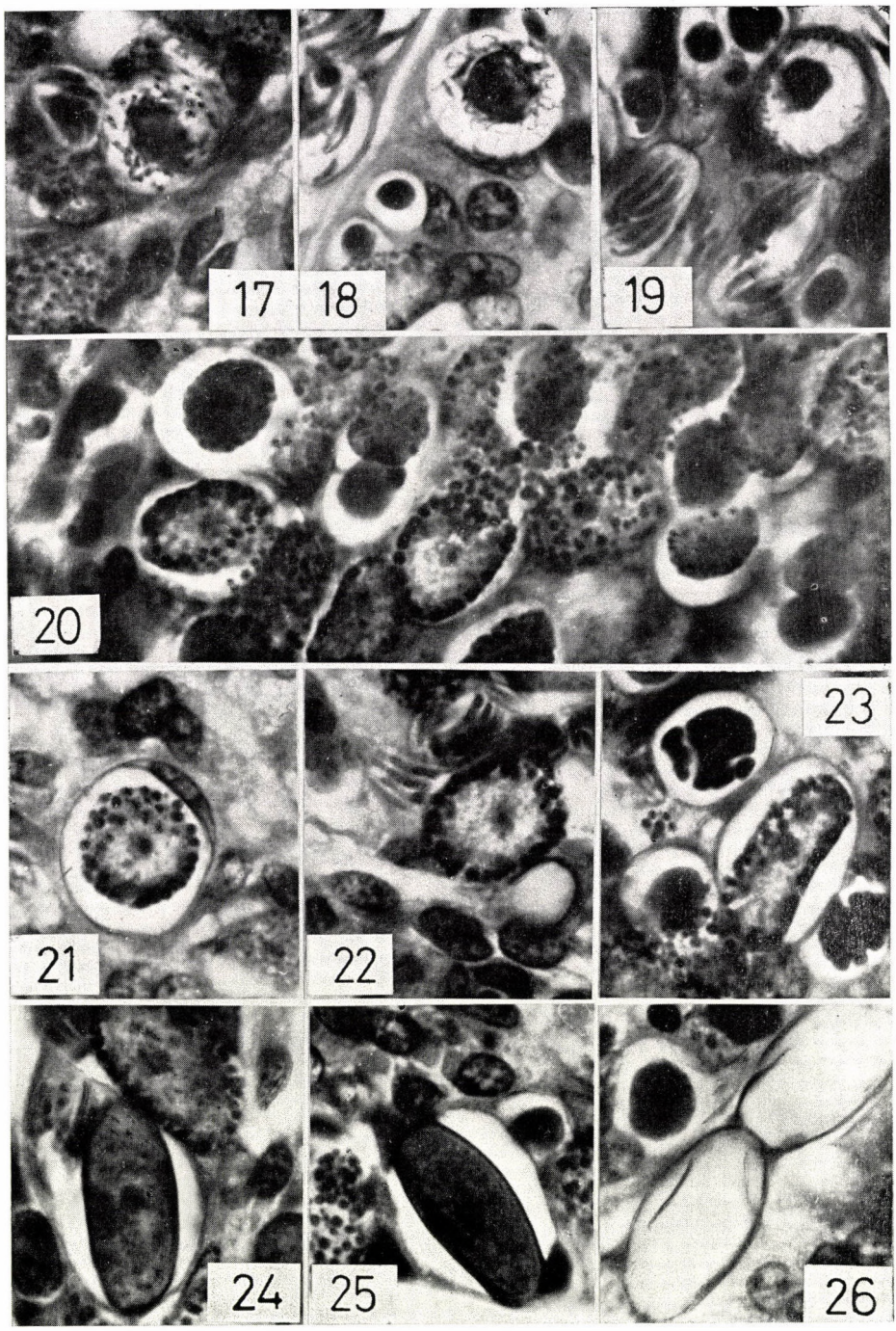
Hares develop a clinical or even lethal coccidiosis under special circumstances, when reinfection is continuously occurring, e. g. in hare breeds, or during transport; this is the reason why lethal coccidiosis is so frequent under such conditions. The mortality is increased by the stress unavoidably imposed upon the animals by transport (Gološin and Tešič, 1963, Gološin *et al.* 1963, and Tešič *et al.* 1963).

In Switzerland, Bouvier (1967) observed in hares intestinal coccidiosis manifested as a severe enteritis. The main causal agents of this disease were *Eimeria leporis* and *Eimeria hungarica*. The small intestinal epithelium was found to desquamate in large patches and many endogenous stages were localizing in the mucosa of the affected intestinal segments.

We observed in carcases of hares submitted from various areas of Hungary a non-haemorrhagic intestinal catarrh and enteritis. The mesenteric vessels were congested and the capillaries of the intestinal wall were injected.

Post mortem examinations of spontaneously died and experimentally

*Figs 17—26. 17—19*, microgamonts with microgametes. × about 1000; *20—22*, Macrogamonts. Note the flattened, bent semilunar nucleus of the host cell in Fig. 21 and the schizonts in Fig. 22. × about 1000; *23—25*, mature macrogametes. Two lobular microgamonts are also visible in Fig. 23. × about 1000; *26*, Indented oocyst walls. × about 1000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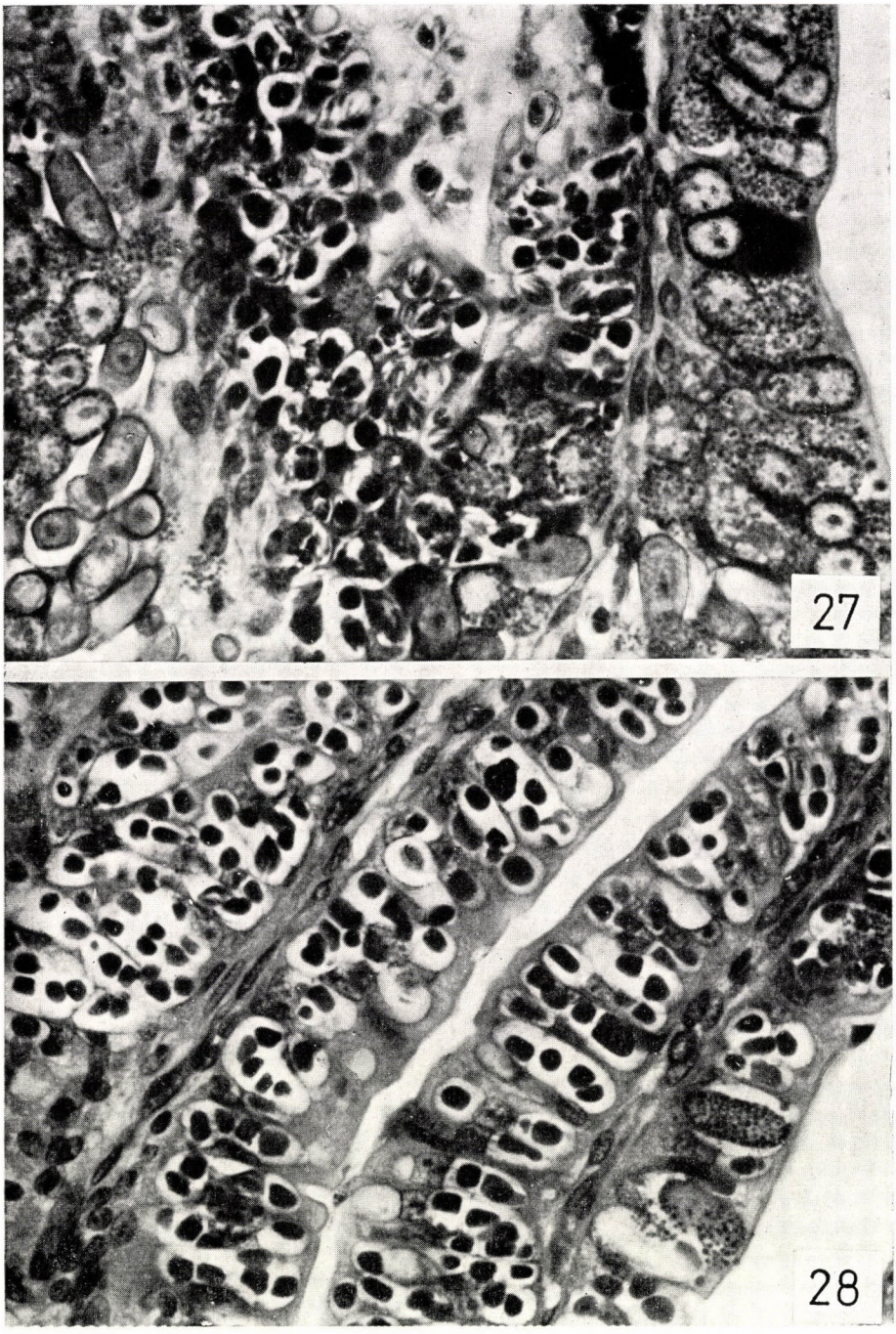
infected hares showed that apart from the above, often slight, inflammatory phenomena, the main gross lesion is represented by greyesh-white foci 1—2 mm in diameter along the entire length of the small intestine, above all in the ileum. The foci are well-visible through the serosa before exposure of the intestine. In the case of massive infections, confluent foci may form gyrus-like irregularly creased areas of a greyish-greenish-white colour, visible through the intestinal wall. In the opened intestine, enlarged intestinal villi are grossly visible in the area of the confluent focal lesions. The capillaries of the intestinal wall are dilated and congested, and scattered groups of granulocytes indicate an inflammatory process. Microscopically the greyish-white mucosal scrapings are found to be packed with different stages, chiefly gamonts and oocysts, of *Eimeria leporis* (Figs 2 and 3).

Studies were carried out on two hares, aged 2.5 and 3 months, which had been infected experimentally with a material containing almost exclusively sporulated *Eimeria leporis* oocysts. Two million oocysts were administered to each animal by the oral route. Both animals had a natural *Eimeria leporis* infestation at the beginning of the experiment; the infection of No. 1 was of a low degree, that of No. 2 of a slightly higher degree.

Hare No. 1 died on the fourth day after experimental infection; unfortunately, its death was noticed too late so that its putrefied organs were no longer suitable for histological processing. Post mortem examination of the emaciated carcase revealed death having been caused by a severe enteritis. Besides, a medium-degree myocardial degeneration and consequent cardiac dilatation occurred. Relatively few *Eimeria leporis* oocysts were found in the intestinal contents; this was not a surprise because the hare died during the schizogonic cycle of endogenous development, supposedly due to lack of a natural infection sufficient to induce an appropriate immune response.

Hare No. 2, which had been more massively infected naturally than hare No. 1, supposedly possessed a firmer protection. To obtain fresh organs for histological examination hare No. 2 was killed in the premortal stage 12 days after the experimental infection.

At post mortem examination of hare No. 2, confluent focal lesions, like those described in the foregoing, were seen along the entire length of the small intestine (Fig. 1), but the large-intestinal mucosa appeared intact. The liver was small, the periportal regions of each of its lobes showed map-like spots dipping slightly below the surface. In the changed areas, of the size of a small coin or smaller, confluent yellowish foci, filled by a caseous substance, were found. Bacteriological examinations were negative; histologically necrosis of the parenchyma was found in the confluent foci. The presence of a young, proliferating connective tissue in the foci indicated an early stage of organization, whereas the heterophilic granulocytes surrounding the foci signified an acute demarcation process.




*Figs 27—28.* Intestinal villi packed with various developmental stages. Chiefly macrogamonts are seen in Fig. 27, but schizogonic stages are also present (centre). Many young gametogonic stages are seen to invade epithelial cells of the villi in Fig. 28. × about 500

Hare No. 2 developed clinical *Eimeria leporis*-coccidiosis and was killed in the premortal stage of the disease. The histological findings confirmed the clinical diagnosis: the schizogonic and gametogonic stages and oocysts of *Eimeria leporis* were present in large numbers within epithelial cells of enlarged small-intestinal villi and, subepithelially, in the connective tissue. The majority of the oocysts found in the intestinal contents were *Eimeria leporis;* after artificial sporulation, out of 700 oocysts eight *Eimeria robertsoni*, three *Eimeria semisculpta*, two *Eimeria europaea* oocysts and one *Eimeria hungarica* oocyst were present.

The liver lesions of hare No. 2 cannot be regarded as specific, being obviously unrelated to coccidiosis and not responsible for the lethal disease. They seem to have arisen from an unknown cause, probably vascular obliteration.

## Discussion

Although *Eimeria leporis* is a well-known common parasite of the hare, the present new data on oocyst morphology, the description of endogenous stages not previously studied and the establishment of the prepatent period are hoped to contribute to the better knowledge of the species.

*Eimeria leporis* being present in more hares without causing any damage, cannot be considered to be obligatorily pathogenic. Under circumstances favouring repeated reinfection, however, the coccidia multiply in the intestine to such a degree that their presence begins to interfere with the processes of digestion and absorption. Such circumstances arise above all in captivity (in hare breeds, during transport, under experimental conditions, *etc.*) and apart from the unusual population density also the stress imposed upon the animals by the artificial environment tends to aggravate the clinical course of coccidiosis. Related practical observations have been substantiated by evidence emerging from experimental infections.

Hares acquire only a low-degree immunity to *Eimeria leporis*-infection. Although the coccidium is very widespread, young and old hares alike can develop a clinical *Eimeria leporis*-coccidiosis under given conditions and in infection experiments massive doses of oocysts proved to be lethal even to those hares which had had a slight natural *Eimeria leporis*-coccidiosis and thus had not been fully susceptible.

Natural *Eimeria leporis*-coccidiosis, although usually mild, is believed to cause economically important losses among hares. The infected animals loose condition and thus become more susceptible to other diseases and fall an easy prey to predatory animals.

## Summary

Studies on carcases of spontaneously died hares *(Lepus europaeus)* submitted to the Wildlife Biology Station and on two experimentally infected young hares showed that massive or repeated infection by *Eimeria leporis* may often cause a lethal coccidiosis. On the basis of these studies, *Eimeria leporis* oocysts were redescribed, the endogenous stages were described in great detail and the prepatent period was established as 7 days. The two young hares, which had had a slight *Eimeria leporis*-coccidiosis, developed a lethal disease on oral infection with two million sporulated *Eimeria leporis* oocysts; from this the conclusion was drawn that the immune response to *Eimeria leporis* is very weak. The low immunogenicity of this species may account for its very frequent occurrence in hares.

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Address of the authors: Dr. László PELLÉRDY, 1071 Budapest, Damjanich u. 56; Dr. Miklós HŐNICH, Dr. László SUGÁR, 2092 Budakeszi, Pf. 13, Hungary

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# EXPERIMENTAL DIARRHOEA IN BABY RABBITS DUE TO ORAL ADMINISTRATION OF HEAT-LABILE ENTEROTOXIN OF *E. COLI* ENTEROPATHOGENIC FOR SWINE

By

F. KUTAS, F. VETÉSI and G. SEMJÉN

Department of Physiology (Head: † Prof. A. KEMÉNY), Department of Pathology (Head: Prof. A. KARDEVÁN), University of Veterinary Science and Veterinary Medical Research Institute (Director: Prof. D. DERZSY), Hungarian Academy of Sciences, Budapest

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The coliform diarrhoea of neonatal pigs is similar to human cholera in several respects: 1. in both diseases, Gram-negative rods proliferate abundantly in the small intestine without invading the tissues; 2. a massive fluid and electrolyte loss occurs through the morphologically intact epithelium into the intestinal lumen, resulting fatal dehydration, hemodynamic disorders and death; 3. a common characteristic of both enteropathogenic *E. coli* and *V. cholerae* is their ability to produce enterotoxin(s); 4. the profuse diarrhoea is attributed to these enterotoxins. Furthermore, an immunological relationship has been demonstrated between the type 2 enterotoxin of *V. cholerae* and the heat-labile *E. coli* enterotoxin (LT) (GYLES and BARNUM, 1969; SMITH, 1972). There is also evidence, that the pathogenesis of the considerable fluid secretion caused by *V. cholerae* or *E. coli* enterotoxins is essentially the same (MOON *et al.*, 1971). It follows from the foregoing that those models which had been developed for studying the diarrhoea-producing effect of *V. cholerae* and its enterotoxin, should be reliable methods for the investigation of the factors involved in the pathomechanism of coliform diarrhoea too. However, the rabbit ileal loop model used for the study of experimental cholera (DE and CHATTERJEE, 1953) has proved to be an unsatisfactory test for demonstrating the enteropathogenicity of *E. coli* strains of porcine origine (SMITH and HALLS, 1967; GITTER, 1967). At the same time, its response to different, particularly heat-labile enterotoxins isolated from these strains, has been fairly consistent (MOON *et al.*, 1970; SMITH and GYLES, 1970; MOON *et al.*, 1971; MOON and WHIPP, 1971; LARIVIÈRE *et al.*, 1972).

Since the description of DUTTA and HABBU (1955), baby rabbits have been extensively used as a tool of experimental cholera. On the other hand, it was only SMITH (1972) who demonstrated that the oral exposure to LT of *E. coli* to which Ent plasmid was transferred from an enteropathogenic porcine *E. coli* strain, caused diarrhoea in 10-day-old rabbits.

On the basis of these findings, experiments were carried out in order to prove that *E. coli* enterotoxin elicited a profuse diarrhoea in baby rabbits resembling to that of suckling piglets. The LT was given to animals, since this type of toxin provoked regularly fluid accumulation in ligated segments of rabbits. Several physiological parameters were recorded thus to examine whether changes in baby rabbits after the oral administration of enterotoxin are of similar value to those observed earlier in pigs with coliform diarrhoea (KUTAS and SZABÓ, 1971).

The final aim of the present study was to develop a method by which the cardinal manifestation of *E. coli* diarrhoea could be produced readily

in intact laboratory animal. It was hoped that the information obtained on this experimental model would be valuable as a step toward the understanding of the pathophysiology of coliform diarrhoea.

## Materials and Methods

### *Experimental animals*

A total of 62 New Zealand white rabbits derived from 15 litters were used. The animals of both sexes were 8 to 12 days old and weighed about 110 to 260 g at time of experiment. The healthy sucking rabbits were separated from their mothers and starved for 6 to 8 hours before the start of experiment.

### *Strains of* E. coli *and preparation of whole-cell lysates*

For preparing whole-cell lysates (WCL), two hemolytic *E. coli* strains were used. One of them, a typical enteropathogenic strain, was kindly submitted by Dr. H. W. SMITH* with the information that No. P99 had been classified serologically as O141 : K85ab,K88ab. The other strain (SP70) isolated from swine with edema disease, belonged to the OK group O141 : K85ab. This strain did not cause fluid accumulation in the ligated intestine of swine. Whole-cell lysates were prepared by the modification of the method of GYLES and BARNUM (1969) as described earlier (PESTI and SEMJÉN, 1973). The whole-cell lysates prepared from enteropathogenic and non-enterotoxic strains are referred subsequently to as heat-labile enterotoxin (LT) and control preparation, respectively.

### *Administration of whole-cell lysates to infant rabbits*

Two groups, experimental and control, were formed. The rabbits in experimental group were treated with LT, those in control group with control preparation. The volume of whole-cell lysates used in both experimental and control rabbits was 5 ml, except for the case of animals heavier than 220 g when 7 ml was given. The preparations were administered *per os* into the stomach using a polyethylene catheter fitted to a hypodermic needle and 10-ml syringe. Administration of preparations occupied 1—2 min., after which the rabbits were kept in separate cages without their mother. Adequate ambient temperature was maintained with the aid of a heat lamp. The rabbits which had not died were sacrificed after 10 to 18 hr.

* A.R.C. Poultry Research Centre, Huntingdon, Great Britain

### *Collection of samples*

Blood samples for determination of standard bicarbonate, glucose and hematocrit were taken from the jugular vein. After this, the rabbits were sacrificed by severance of the carotid artery. The heparinized blood samples collected in this way were centrifuged and the plasma was used for determination of urea, plasma protein and osmolality.

The urine sample necessary for the determination of pH was obtained immediately after extermination from the bladder. At the same time, samples of 1 to 2 ml of large intestinal contents were taken anaerobically from the experimental animals and the pH, standard bicarbonate and osmolality were determined. The amount of intestinal fluid found in the large intestine of control animals was insufficient for analysis. In other cases, the gut was used for determination of total "intestinal fluid". After having measured the weight of the whole intestinal tract, it was slit up longitudinally, dried with filter paper and weighed again. The amount of intestinal fluid was calculated as difference of the two weights.

### *Analytical methods*

The majority of the analytical methods used for determination of blood constituents have been described elsewhere (KUTAS and SZABÓ, 1971). Blood sugar was determined by the o-toluidine method (Biochemica Test Combination, Boehringer Mannheim GMBH). The osmolality of plasma and intestinal fluid was estimated by freezing point depression with a Knauer Osmometer (Germany). The pH of intestinal fluid and urine was measured in duplicate at 38 °C with a capillary electrode (E 5021a, Radiometer, Denmark). The standard bicarbonate of intestinal fluid was measured by the equilibration method of ASTRUP *et al.* (1960). At the calculation the modified nomogram of SIGGAARD ANDERSEN was used (KIENLE, 1969).

### *Bacteriological methods*

At post-mortem examination specimens were taken aseptically from the different segments of intestine and cultured on blood agar plates for accidental contamination with hemolytic *E. coli* strains.

### *Statistics*

The statistical significance of difference between mean values was calculated by Student's *t* test.

## Results

### *Clinical signs and symptoms*

The results shown in Table I indicate that 32 of the 41 rabbits given LT developed profuse diarrhoea. Three animals showed mild diarrhoea and 6 rabbits failed to develope diarrhoea but 5 of these exhibited fluid accumulation at autopsy. One animal showed neither diarrhoea nor fluid accumulation. It is interesting to record that diarrhoea already began in some experimental animals 3 1/2—4 hr. after administration of enterotoxin.

The rabbits that developed severe shock were unable to move. Their skin became cool and cyanotic. Loss of tissue turgor and elasticity was also observed. Diarrhoea was continuous until the moribund state. Death occurred as early as 10 hr. or as late as 20 hr. after administration of LT. Ten rabbits died and 4 were sacrificed in moribund condition.

**Table I**

Response of rabbits to oral administration of *E. coli* enterotoxin listed on the basis of clinical and post-mortem signs

| Group | Number of rabbits | | | | | | |
|---|---|---|---|---|---|---|---|
| | Challenged | Diarrhoea | | | Fluid accumulation in intestine | | |
| | | Severe | Mild | No | Severe | Mild | No |
| Experimental (LT) | 41 | 32 | 3 | 6 | 35 | 5 | 1 |
| Control | 21 | — | — | 21 | — | — | 21 |

Other diarrhoeic rabbits did not develope shock during the time of observation and showed only mild clinical symptoms of dehydration. Twenty-seven animals were exterminated in relatively fair condition 9 to 18 hr. after administration of enterotoxin.

The control rabbits sacrificed 8 to 24 hr. after treatment did not develop any diarrhoeal symptoms.

### *Post-mortem findings*

At autopsy, when the abdomen of rabbits given LT was opened, the distended large intestine appeared prominently (Fig. 1). The stomach was also markedly dilated and filled with coagulated milk and occasionally some excess mucus. The stomach wall showed congestion: the dilatation of gastro-epiploic blood vessels could be observed. After the extermination of affected animals, no or only slight peristaltic waves were noticed along the walls of the gut. The small and large intestine, with the exception of duodenum, was distended and contained a large amount of watery or mucinous-watery, sometimes slightly reddish fluid. The cecum, colon and mesocolon of the diar-

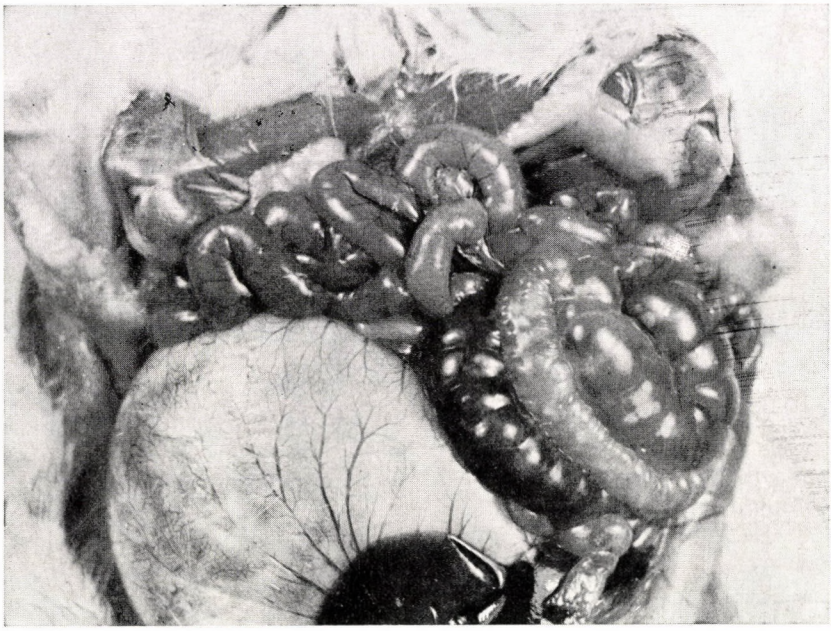

*Fig. 1.* The gastrointestinal tract of 11-day-old, infant rabbit 14 hr. after the administration of 5 ml LT preparation

rhoeic rabbits were characterized with mild hyperemia and submucosal edema. In the relatively mild cases, the excessive amount of liquid in the colon and rectum contained also preformed feces. Macroscopic hemorrhage or necrosis could not be observed in the stomach and intestine. The urinary bladder was empty or contained only a small amount of urine.

At necropsy of control rabbits no pathological findings were observed. Bacteriological examination of the intestine revealed that none of the experimental and control rabbits were contaminated with hemolytic *E. coli* strains.

**Table II**

Data relating to loss of body water following LT administration

| Parameter | | Experimental (LT) | | | Control | | |
|---|---|---|---|---|---|---|---|
| | | n | Mean | S. E. | n | Mean | S. E. |
| Gain (+) or loss (−) of body weight | g | 41 | −11.51 | ±0.97 | 20 | +1.25 | ±0.79 |
| | per cent* | 41 | −6.70 | ±0.57 | 20 | +0.86 | ±0.44 |
| Amount of intestinal content | g | 20 | 8.09 | ±0.51 | 10 | 1.08 | ±0.21 |
| | per cent* | 20 | 5.25 | ±0.46 | 10 | 0.80 | ±0.14 |

* Expressed in per cent of body weight at beginning of experiment

Tissues for histopathologic and histochemical examination were also taken. The results of these investigations will be published in a following paper.

### *Laboratory analysis of body fluids*

*Water balance.* The results in Table II show that an average loss of 6.70 per cent of the body weight was observed in the experimental group. Since acute losses of weight reflect changes in amount of total body water, a significant dehydration in the experimental rabbits can be established. In addition, the accumulated fluid found in the intestinal tract of affected animals also represents a loss of the body water. Consequently, the amount of intestinal fluid, 5.25 per cent of body weight can be added to the data of weight loss. This means that altogether about 12 per cent of body weight can be considered as water deficit which represents a very severe form of water depletion.

As shown in Table III the laboratory data clearly support the clinical finding of dehydration. Significant increases of hematocrit and plasma protein values indicated hemoconcentration due to the diminution of plasma volume. In order to determine the type of dehydration, the osmolality of plasma was also measured. No significant differences were observed between the osmolality values in the plasma of experimental and control animals: 294.86 — and 297.75 mOsm/l, respectively. This refers to the loss of salt and water in isotonic ratio, consequently the osmolality in plasma remains unchanged, in spite of the reduction of the extracellular water (isotonic dehydration).

The intestinal contents of the affected rabbits were near-isotonic with blood plasma. The ratio of $\text{osmolality}_{\text{plasma}}$ to $\text{osmolality}_{\text{intestinal fluid}}$ was found to be 1.13.

The increase of plasma urea observed in experimental rabbits is also characteristic of the advanced form of dehydration.

**Table III**

Blood analysis

| Constituent | Experimental (LT) | | | Control | | |
|---|---|---|---|---|---|---|
| | n | Mean | S. E. | n | Mean | S. E. |
| Hematocrit, % | 27 | 58.85 | ±2.10 | 17 | 42.35 | ±1.16 |
| Total protein, g/100 ml plasma | 15 | 7.51 | ±0.43 | 13 | 5.19 | ±0.31 |
| Urea, mg/100 ml plasma | 15 | 113.27 | ±21.52 | 13 | 48.62 | ±5.73 |
| Standard bicarbonate, mEq/l plasma | 28 | 15.98 | ±1.40 | 17 | 23.85 | ±0.41 |
| Osmolality, mOsm/l plasma | 7 | 294.86 | ±4.75 | 8 | 297.75 | ±7.62 |
| Glucose, mg/100 ml blood | 16 | 149.44 | ±12.81 | 11 | 135.27 | ±9.20 |

*Acid-base balance.* As shown in Table III, after administration of LT a significant fall of standard bicarbonate was observed in the blood of rabbits. It should be noted that at terminal state very low values were found: in all 4 rabbits exterminated in moribund condition the standard bicarbonate values were less than 10 mEq/l. The metabolic acidosis can be explained with the intestinal loss of bicarbonate. Table IV shows that the average bicarbonate concentration of intestinal fluid (35.26 mEq/l) was more than twice as high as that of plasma (15.98 mEq/l).

**Table IV**

Analysis of large intestinal contents

| Constituent | Experimental (LT) | | |
|---|---|---|---|
| | n | Mean | S. E. |
| pH | 18 | 7.19 | $\pm$ 0.01 |
| Standard bicarbonate, mEq/l | 31 | 35.26 | $\pm$ 2.10 |
| Osmolality, mOsm/l | 9 | 259.22 | $\pm$ 6.82 |

In spite of the fact that the intestinal content of the enterotoxin-treated rabbits was rich in bicarbonate, its mean pH was found relatively low, only 7.19. This can be explained with the high carbon dioxide tension present in the intestinal fluid. $P_{CO_2}$ values were found between 66 and 136 mmHg in 16 of the total 20 samples collected. In 4 rabbits the intestinal carbon dioxide tension was higher than 150 mmHg.

The values of urine pH were of the similar magnitude in both groups. Control: pH 6.6 $\pm$ 0.21 (n = 19), experimental: 6.5 $\pm$ 0.37 (n = 7).

Statistical analysis of the data are presented in Table V.

**Table V**

Statistical analysis of the mean values

| Determination | Direction of change in the mean | *P* (less than the value shown) Experimental vs. Control |
|---|---|---|
| Body weight | Decrease | 0.001 |
| Intestinal water | Increase | 0.001 |
| Hematocrit | Increase | 0.001 |
| Total protein, plasma | Increase | 0.001 |
| Urea, plasma | Increase | 0.02 |
| Standard bicarbonate, plasma | Decrease | 0.001 |
| Osmolality, plasma | Slight decrease | NS* |
| Glucose, blood | Slight increase | NS |

* NS, not significant

## Discussion

In accordance with the findings of SMITH (1972), our experiments on infant rabbits clearly proved that these animals are highly susceptible against LT elaborated by *E. coli*. It has been demonstrated that when LT of enteropathogenic *E. coli* strains of porcine origin comes into direct contact with intestinal mucosa, there follows a hypersecretion of intestinal fluid. Fluid accumulation, diarrhoea develops and in severe case the animal dies. In contrast, after the administration of WCL of non-enterotoxigenic *E. coli* strains no pathological signs were observed. On the basis of these results it may be concluded that enterotoxin is responsible for diarrhoea observed in our experimental rabbits.

The toxin produces changes in ion flux across the epithelium of the intestine. Enterotoxins elaborated by both *V. cholerae* and *E. coli* stimulate intestinal adenyl cyclase and the resulting increase in cellular adenosine-3':5'-monophosphate (cAMP) is a signal that produces changes in ion transport (AL-AWQATI *et al.*, 1972). In our experiments an enterotoxin-induced copious secretion of salt and water into the infant rabbits' intestinal lumen could be observed. It has been demonstrated that severe diarrhoea developed within 3 to 4 hr. following orogastric administration of *E. coli* enterotoxin. A near-isotonic, bicarbonat-rich fluid was accumulated mainly in the large intestine.

The loss of isotonic intestinal fluid took place very quickly and caused isotonic dehydration. The average water deficit of about 12 ml per 100 g body weight observed in our experiment indicated very severe form of dehydration, since the normal plasma volume for 11-day-old rabbits has been estimated at 5.17ml per 100 g of body weight (LITTLE, 1970). The rapid fall of the volume of extracellular water led to circulatory insufficiency which became the prominent feature of the clinical picture. Circulatory collapse developed in a few hours after the first symptoms of diarrhoea. In this shock phase uremia also developed, as the glomerular filtration rate decreased. Diarrhoeal loss of alkaline fluid depleted the bicarbonate pool of extracellular fluid and caused metabolic acidosis. It is interesting to compare the bicarbonate level of intestinal fluid to that of host plasma. As seen previously, in the shock phase there was over two times the concentration of bicarbonate in the intestinal fluid as there was in the plasma. If its absorption took place, the bicarbonate pool present in the intestinal content could at least partially compensate the severe form of acidosis in the extracellular fluid.

It should be pointed out that the effectiveness of renal regulation is limited against the disturbances of the water and acid-base balance. The neonatal rabbit, like the human baby, piglet, puppy etc. has a poorly developed renal function compared with the adult. Such "renal immaturity" is evident in the neonate's inability to produce very concentrated urine when dehydrated

(DALTON, 1968). The results of our experiments indicate an inadequate renal compensation, since no significant difference was observed between the urine pH values in acidotic and normal animals.

The changes seen after the administration of *E. coli* enterotoxin in infant rabbits resemble those found by us in naturally occurring form of mucoid enteritis associated with *E. coli* in rabbit (VETÉSI and KUTAS, 1973), as well as in that of enteric colibacillosis in piglet (KUTAS and SZABÓ, 1971). However, there were certain differences in the clinical and post-mortem picture. Excessive fluid accumulation and distension of large intestine were more characteristic of the disease observed in rabbit. Such differences can be explained with the different anatomical and physiological features of digestive system in rabbit and swine. One of these is that, in contrast with the pig, the rabbit vermiform appendix is a secretory organ. The volume of fluid produced by the appendiceal secretion of adult rabbit has been estimated 20—80 ml per day (BLACKWOOD *et al.*, 1973). At any rate, the informations obtained by means of infant rabbit model seem pertinent to an understanding of the mechanism of diarrhoea caused by *E. coli* and may help to clarify the pathophysiology of disease.

The response of ligated intestinal loops of pig jejunum and rabbit ileum have been widely used for study of *E. coli* enterotoxin. In our opinion, the infant rabbit model has several advantages when it is compared to these experimental systems:

(1) Because of the antimicrobial action of stomach during the suckling period, the stomach and small intestine of baby rabbit are practically sterile (SMITH, 1965; SMITH, 1966). Thus, the response following administration of enterotoxin is not disturbed by the interaction of intestinal microflora and the different immunological background.

(2) No preparatory and surgical pre-treatment is necessary.

(3) The intestinal tract remains unobstructed.

(4) While the ligated intestinal loop preparation is essentially a static system, the infant rabbit model offers an opportunity to study the intact animal.

(5) Baby rabbits are readily available and less expensive than either pigs or adult rabbits.

**Acknowledgement**

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**Summary**

The *E. coli* enterotoxin-induced pathophysiological changes were studied in infant rabbits and compared to the response of control animals treated with whole-cell lysate from non-enterotoxigenic strain.

It has been shown that heat-labile enterotoxin produced from enteropathogenic *E. coli* strain of porcine origin produces diarrhoea, causing a characteristic clinical response and post-mortem change, while the whole-cell lysate from non-enterotoxigenic *E. coli* strain fails to do so.

The changes after administration of enterotoxin were closely resembling those observed in the naturally occurring form of rabbit mucoid enteritis and coliform diarrhoea of piglets: increased fluid secretion into the intestinal lumen, diarrhoea, severe dehydration, metabolic acidosis and uremia.

The volume and composition of fluid accumulated in intestine was also studied. When compared to host plasma, this fluid was high in bicarbonate and nearly isoosmolar.

The infant rabbit has been suggested as a suitable experimental model for the study of effect of *E. coli* enterotoxin.

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Address of the authors: Dr. Ferenc KUTAS, Dr. Ferenc VETÉSI, 1078 Budapest, Landler J. u. 2; Dr. Gábor SEMJÉN, 1143 Budapest, Hungária krt. 21, Hungary.

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# COMPARATIVE STUDY OF SWINE *MYCOPLASMA* SPECIES

By

L. STIPKOVITS, D. SCHIMMEL and Lea VARGA

Veterinary Medical Research Institute (Director: D. DERZSY) Hungarian Academy of Sciences, Budapest and Institute for Bacterial Diseases of Animals, Jena (Director: TH. HUBRIG), Academy of Agricultural Sciences of the GDR

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Recent world-wide interest in mycoplasma research has resulted in a marked increase in the number of known species from various hosts. Among the many new species several pathologically important ones have been detected (HAYFLICK, 1969), which differ from the earlier descriptions, but their systematic position is still unclear.

The main problem of mycoplasma research is that the test materials usually contain more than one species of which some are saprophytic. Species differentiation is rendered difficult by the variable nutritional requirements and growth ability of the various strains as well as by the inadequate knowledge of the biological properties of certain species.

The clarification of the aetiological role of mycoplasmas is preconditioned by the identification and close study of the various species involved in the pathological processes. Detailed investigation and comparison of the known species and the elaboration of standard procedures, especially of simple biochemical tests, for species identification, are therefore imperative. Related studies have been supported by the competent teams of the World Health Organization (WHO).

This paper is a report of comparative studies on the commonly occurring swine mycoplasma species *Acholeplasma granularum* (SWITZER, 1964), *Mycoplasma hyorhinis* (DINTER *et al.*, 1965), *M. hyopneumoniae* (syn.: *M. suipneumoniae*) (GOODWIN *et al.*, 1965; MARE and SWITZER, 1965) *M. hyosynoviae* (ROSS and DUNCAN, 1970) and *A. laidlawii* (SCHIMMEL and PUSTOVAR, 1971).

## Materials and Methods

*Strains.* The following reference strains, supplied from the FAO/WHO International Reference Centre for Animal Mycoplasmas (Institute of Medical Microbiology, University of Aarhus, Aarhus, Denmark), were used:

*A. granularum* (Friend)
*M. hyorhinis* PG 29

*M. suipneumoniae* "J" (ATCC 25934)
*M. hyosynoviae* AMRC/C 104
*A. laidlawii* A PG 8
*A. laidlawii* B F—8

All strains were subcultured three times after a previous filtration and the third subculture was distributed in test tubes and stored at —70 °C. In all experiments, the third subculture was used as the initial material.

*Media*

*BE medium* (one lot)

| | | |
|---|---|---|
| Heart Infusion Broth (DIFCO, USA), according to producer's prescriptions | 90 | ml |
| Inactivated horse serum | 20 | ml |
| Yeast extract (prepared as described by HAYFLICK) | 10 | ml |
| 10% thallium acetate solution (Fischer Scientific Co., USA) | 1 | ml |
| 1% 2,3,5 triphenyl-tetrazolium-chloride (MERCK, GFR) | 1 | ml |
| 0.2% deoxyribonucleic acid (from calf thymus, SIGMA, USA) | 1.2 | ml |
| 200,000 IU/ml penicillin | 0.25 | ml |

In solid media, the heart infusion broth was replaced by heart infusion agar.

All examined strains except *M. suipneumoniae* were grown in the BE medium. *M. suipneumoniae* was cultured in the media proposed by GOODWIN and PRYOR (1970), FRIIS (1971) and GOURLAY and LEACH (1970). The same media were used in the biochemical and resistance tests, after a slight modification if required.

*Determination of the growth curves*

The strains were grown on streak plates of BE agar for 3—4 days, after which agar blocks, each bearing a single colony, were excised and transferred to 2 ml BE broth or to broth containing glucose or arginine, but no tetrazolium chloride (see below). The blocks were homogenized in the broth and germ counts were determined in terms of colony-forming units (CFU). The broth cultures were then incubated for 10 days, during which CFU were determined daily. The "J" strain of *M. suipneumoniae* was grown on Goodwin's agar inoculated with 0.2 ml of a 1 : 100 diluted broth culture and 0.5 × 0.5 cm

blocks of Goodwin's agar were transferred to 2 ml each of Goodwins's, Friis's and Gourlay—Leach's medium containing broth for growth curve determination. The latter was in this instance not based on CFU, but on the determination of the highest culture dilution that changed the colour of the broth culture in 7 days when added to it in 0.1 ml amount (Figs 1, 2).

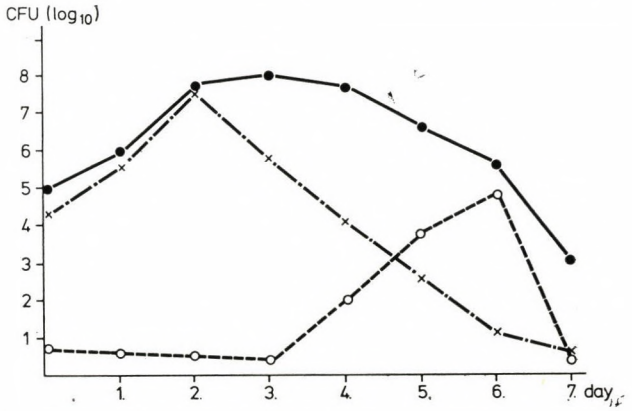


*Fig. 1.* The growth curve of swine *Mycoplasma* species. ——— *M. hyorhinis* PG 29; — · — *M. hyosynoviae;* — — — *M. suipneumoniae*

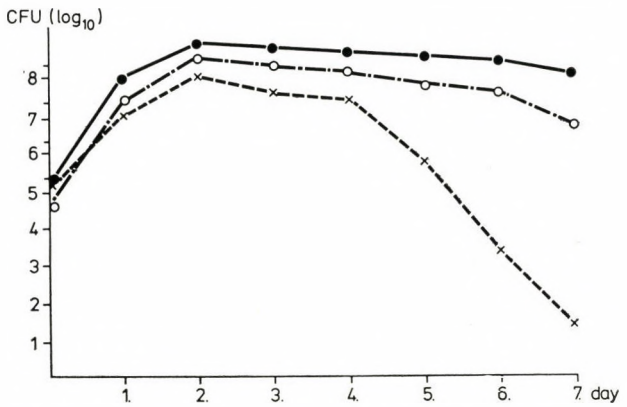


*Fig. 2.* The growth curve of swine *Acholeplasma* species. ——— *A. Laidlawii* A PG 8; — · — · — *A. laidlawii* B PG 9; — — — *A. granularum* (Friend)

### *Determination of the culturing conditions*

The broth cultures of the strains were diluted at tenfold steps up to $10^{-6}$ and from each dilution 0.01 ml was inoculated onto 4 BE plates, from *M. suipneumoniae* to 4 Friis's agar plates. The first series of agar plates were incubated under aerobic conditions, the second in an atmosphera of 95% nitrogen + 5% carbon dioxide, the third in nitrogen atmosphere, for 10 days

at 37 °C. The fourth series was incubated with a burning candle placed in the incubating jar. Growth results were read every other day according to the score evaluation system of ERNØ *et al.* (1967) in which the index characterizing the growth properties of the strain is calculated from the scores indicating colony outgrowth with regard to the degree of dilution of the inoculum and time of inoculation.

### *Examination of biochemical properties*

Decomposition of carbohydrates (glucose under aerobic and anaerobic conditions, mannose, sucrose, galactose, mannitol, sorbitol, xylose), arginine and urea was examined in liquid BE medium containing 1 ml Bacto PPLO serum fraction (DIFCO), 5 ml 10% substrate solution and 5 ml 60 mg/100 ml phenol red, but neither tetrazolium chloride nor horse serum. The heart infusion broth and yeast extract were treated with glucose oxidase, peroxidase and arginase before added to the medium. The carbohydrate-containing media and the basal BE medium (control) free from substrate were adjusted to pH 7.8, the arginine- and urea-containing media and the corresponding basal (control) BE medium to pH 7.3. The reaction was read on the basis of colour change, daily for the first 5 days and every other day subsequently. The reaction was regarded as positive if the fall or rise of the pH reached at least 0.5 unit in comparison to the pH of inoculated and uninoculated substrate-free control media.

The reducing capacity of the strains was tested under aerobic and anaerobic conditions in BE broth containing 1 or 5 ml 1% triphenyl-tetrazolium chloride and 1% Bacto PPLO serum fraction, in broth containing rabbit meat extract (ALUOTTO et al., 1970), and in BE broth containing 5 ml 1% potassium tellurite and 0.24 ml 0.1% methylene blue solution. Reduction of the first two substrates was indicated by a red colour change, that of the third substrate by discoloration of the cultures.

### *Inoculation technique*

Substrate-containing and substrate-free media were inoculated each with a single colony obtained from a 3—4 day agar culture of the strain tested and 0.1 ml from each tube culture was transferred to an identical medium 2—4 days later, depending on the growth curve. In the meantime BE streak plates were prepared to check mycoplasmal growth. Tube cultures to be incubated under anaerobic conditions were inoculated with material from cultures grown aerobically on medium containing the same substrate. Each examination was made in three replicas. To exclude bacterial contami-

nation, positive reactions were always checked by streaking on blood agar plates.

The hydrolysis of aesculin was examined by the method of WILLIAMS and WITTLER (1971), phosphatase activity and serum digestion were tested according to ALUOTTO *et al.* (1970), film and spot formation and cholesterol requirement according to EDWARD (1971, 1950). Arbutin decomposition was examined on BE agar, containing 1.2 ml 10% arbutin (hydroquinone-beta-D-glycopyranoside, SIGMA) and 1.2 ml 5% ferric citrate, but no triphenyl-tetrazolium-chloride. The reliability of the tests was checked by means of the test strains proposed by ALUOTTO *et al.* (1970).

### *Examination of sensitivity to sodium chloride, sodium azide, methylene blue, Tween 80, sodium taurocholate, pH 5.5 and pH 9.5*

These tests were carried out on modified BE media in which triphenyl tetrazolium chloride was replaced by 3.6 g sodium chloride, 6.0 ml 10% sodium taurocholate, 15 ml 1% sodium azide, 2.5 ml 10% Tween 80 or 2.4 ml 1% methylene blue per lot. Sensitivity to pH was examined on the usual BE agar plates with pHs adjusted to 5.5 and 9.5, respectively. The freshly prepared solid test media and the BE basal medium were inoculated with 0.01 ml of undiluted and $10^{-3}$-diluted broth culture, respectively. The test plates were incubated for 14 days, and if growth was observed, the respective agar block was excised and transferred to an identical medium. If no growth occurred in 10 days, an excised agar block was transferred to BE broth to check the survival of mycoplasmas. The test was repeated several times.

### *Examination of sensitivity to sodium polyanethol sulphonate, digitonin and antibiotics*

0.01 ml of culture dilutions containing $10^5$ CFU were transferred to solid BE medium from which the penicillin component was omitted if antibiotic sensitivity was tested, and discs containing the test substances were placed on the surface of the medium. The discs were soaked with 0.02 ml 5% sodium polyanethol sulphonate (Hoffman—La Roche and Co., Switzerland) (KUNZE, 1971) or 0.02 ml 0.01% Digitonin (Merck, GFR) dissolved in ethanol, and were dried for 24 hours. Antibiotic sensitivity was tested by means of ready-to-use discs (HUMAN, Budapest) containing 10 $\mu$g erythromycin, 30 $\mu$g kanamycin or 15 $\mu$g polymyxin B. The radii of the inhibition zones were measured after 3—4 days; in the case of *M. suipneumoniae*, the incubation time was 10 days.

### *Examination of* M. suipneumoniae

The biochemical and resistance tests were carried out on cultures grown in the Gourlay-Leach medium. The lactalbumin hydrolysate, Hartley's Digest Broth and foetal calf serum constituents of the media used for testing carbohydrate, arginine and urea decomposition, were pretreated with glucose oxidase, peroxidase and arginase. The quantity of substrates was the same as in the BE test media. Glucose and phenol red were omitted from those media which were used for testing the hydrolysis of aesculine and arbutin, and the serum component was inactivated for one hour. Cultivation of the strains and inoculation of the test media were performed as previously described.

## Results

All the strains except *M. suipneumoniae* grew readily in solid and liquid BE medium, and M. suipneumoniae grew well in solid or liquid Goodwin's, Friis's and Gourlay-Leach's media. The growth rate of the strains can be seen in Figs 1 and 2; the acholeplasmas grew as a rule faster than the mycoplasmas. In cultures of the *A. laidlawii* A PG 8 and *A. laidlawii* B F-8 strains, the number of CFU was high until the 7th day. In *A. granularum* (Friend) cultures the CFU decreased in number from the 5th day on. The counts of *M. hyorhinis* PG 29 were high for four days, whereas those of *M. hyosynoviae* AMRC/C104 began to decrease abruptly after reaching maximum CFU on the third day. The *M. suipneumoniae* "J" strain was characterized by a slow growth, reaching maximum titre on the 7th day, but this maximum was by 2—3 logarithmic units lower compared to the titres of other strains. The titre fell abruptly after the 7th day. In liquid media containing glucose or arginine, the growth rate was the same as in the BE medium during the initial days of incubation, but later the reduction in the CFU value was more pronounced than in the substrate-free media.

Cultures of the *A. laidlawii* A PG 8 and *A. laidlawii* B F-8 strains showed the colour change indicative of glucose decomposition already on the first day and the acholeplasma counts scarcely changed until the 6th day. The cultures of the *A. granularum* (Friend) strain began to take on a yellow colour after 3—4 days, and the acholeplasma counts tended to reduce from the 4th day. With *M. hyorhinis* PG 29, the colour change indicating carbohydrate decomposition remained indistinct throughout, whereas with *M. hyosynoviae*, shift of the pH to the alkaline region owing to arginine decomposition began to appear on the third and on the very day the mycoplasma counts began to decrease.

Peak values of growth under different conditions of culturing are summarized in Table I. The *M. suipneumoniae* strain grew exclusively under anaerobic

**Table I**

Growth of various *Mycoplasma* and *Acheloplasma* species under different atmospheric conditions

| Strain | | Atmosphere | | | |
|---|---|---|---|---|---|
| | | $N_2$ | 95% $N_2$ + 5% $CO_2$ | "candle jar" | Air |
| *M. granularum* (Friend) | Index | 5.0 | 5.2 | 5.0 | 5.2 |
| | CFU | $2.0\times10^8$ | $8.0\times10^7$ | $5.0\times10^7$ | $9.0\times10^7$ |
| *M. hyorhinis* PG 29 | Index | 4.0 | 4.2 | 5.0 | 4.4 |
| | CFU | $2.0\times10^7$ | $6.0\times10^7$ | $7.8\times10^7$ | $6.0\times10^7$ |
| *M. hyosynoviae* AMRC/C 104 | Index | 4.0 | 4.2 | 4.8 | 4.0 |
| | CFU | $2.0\times10^6$ | $4.2\times10^6$ | $4.9\times10^6$ | $2.7\times10^6$ |
| *M. suipneumoniae* "J" ATCC 25934 | Index | 3.2 | 3.6 | 3.4 | 0.6 |
| | CFU | $1.0\times10^5$ | $4.0\times10^5$ | $3.6\times10^5$ | n.t. |
| *A. laidlawii A* PG 8 | Index | 6.8 | 6.0 | 7.4 | 6.6 |
| | CFU | $3.5\times10^7$ | $1.0\times10^7$ | $1.0\times10^7$ | $2.5\times10^7$ |
| *A. laidlawii B* F—8 | Index | 6.2 | 6.2 | 6.2 | 6.8 |
| | CFU | $1.0\times10^7$ | $2.0\times10^7$ | $2.1\times10^7$ | $5.0\times10^7$ |

conditions, whereas the other strains grew equally well in aerobic and anaerobic conditions.

Among the examined strains *M. hyosynoviae*, *M. hyorhinis* and *M. suipneumoniae* were found to require cholesterol. The *A. laidlawii* A and B strains and *A. granularum* grew readly also in cholesterolfree medium. The above three mycoplasma strains were sensitive to sodium polyanethol sulphonate and digitonin, whereas the remaining were not (Table II).

**Table II**

Cholesterol requirement and sodium polyanetholsulphonat and digitonin sensitivity of the examined strains

| Strain | Cholesterol requirement | Sensitivity to | |
|---|---|---|---|
| | | sodium polyanethol-sulphonate | digitonin |
| *A. granularum* (Friend) | — | — | — |
| *M. hyorhinis* PG 29 | + | 8.0* | 8.0 |
| *M. hyosynoviae* AMRC/C 104 | + | 5.0 | 6.0 |
| *M. suipneumoniae "J"* (ATCC 25934) | + | 15.0 | 7.0 |
| *A. laidlawii A* PG 8 | — | — | — |
| *A. laidlawii B* F—8 | — | — | — |

* The figures designate the radius of the inhibition zone in mm

The following strains displayed a glucose-decomposing activity: *A. laidlawii* A PG 8, *A. laidlawii* B F-8, *A. granularum* (Friend), *M. hyorhinis* PG 29 and *M. suipneumoniae* "J". The glucose decomposing activity of the latter two strains was, however, less distinct compared to that of the others and the fall of the pH scarcely exceeded 0.5 unit. As to the other carbohydrates, xylose and galactose were weakly decomposed by *M. suipneumoniae* and aesculin was decomposed exclusively by the *A. laidlawii* strains, but mannose, mannitol, sucrose, sorbitol, cellobiose and arbutin were not broken down by any of the examined strains, nor was urea.

Arginine was decomposed by *M. hyosynoviae*. Phosphatase activity was positive with *M. hyorhinis*, film and spot formation with *M. hyosynoviae*. All examined strains reduced triphenyl-tetrazoliumchloride under anaerobic conditions and *M. suipneumoniae* reduced it also under aerobic conditions. The latter strain was the only one which reduced potassium tellurite. Methylene blue was reduced by all strains except *M. hyosynoviae* under anaerobic conditions (Table III), but by none under aerobic conditions. Serum digestion was not found with any strain.

**Table III**

Biochemical properties of the examined strains

| Test | Strains | | | | | |
|---|---|---|---|---|---|---|
| | *A. gran.* (Friend) | *M. hyorh.* PG 29 | *M. hyosynoviae* (AMRC/C 104) | *M. suipn.* "J" (ATTC 25934) | *A. laidl.* A PG 8 | *A. laidl.* B F–8 |
| Glucose | | | | | | |
| aerobic | ++ | + | – | + | + | + |
| anaerobic | ++ | – | – | + | + | + |
| Galactose | – | – | – | + | – | – |
| Xylose | – | – | – | + | – | – |
| Aesculin | – | – | – | × | + | + |
| Arginine | – | – | ++ | – | – | – |
| Triphenyltetrasolium chloride | | | | | | |
| aerobic | – | – | – | + | + | + |
| anaerobic | + | + | + | + | + | + |
| Potassium tellurite | | | | | | |
| aerobic | – | – | – | + | – | – |
| anaerobic | – | – | + | + | – | – |
| Methylene blue | | | | | | |
| aerobic | – | – | – | – | – | – |
| anaerobic | ++ | + | | (+) | + | + |
| Phosphatase activity | – | + | – | – | (+) | – |
| Film and spot formation | – | – | + | – | – | – |

Symbols used: (+) mild reaction; + moderate reaction; ++ strong reaction; – negative; × no growth

As to the resistance tests, *M. suipneumoniae* was found to be sensitive to all substances and antibiotics tested. *M. hyorhinis* behaved similarly, but erythromycin inhibited its growth to a lesser degree than that of *M. suipneumoniae*. *M. hyosynoviae* was resistant to sodium azide and erythromycin, *A. granularum* to sodium azide, pH 9.5 and polymyxin B and the *A. laidlawii* A and B strains resisted sodium azide, Tween 80, sodium chloride, polyxin B and pHs 5.5 and 9.5. (Table IV)

**Table IV**

Resistance tests of the examined strains

| | *A. gran.* (Friend) | *M. hyorh.* PG 29 | *M. hyosynoviae* (ÁMRC/C 104) | *M. suipn.* "J" (ATCC 25934) | *A. laidl.* A PG 8 | *A. laidl.* B F—8 |
|---|---|---|---|---|---|---|
| Sodium chloride | S | S | S | S | R | R |
| Methylene blue | S | S | S | S | S | S |
| Tween 80 | S | S | S | S | R | R |
| Sodium azide | R | S | R | S | R | R |
| pH 5.5 | S | S | S | S | R | R (1) |
| pH 9.5 | R | S | S | S | R | R |
| Sodium taurocholate | S | S | S | S | S | S |
| Erythromycin | 31 | 6.0 | 0 | > 30 | 22.0 | 24.0 |
| Polymyxin B | 0 | > 25 | > 25 | > 30 | 0 | 0 |
| Kanamycin | 16 | > 20 | 25 | > 30 | 10.0 | 9.0 |

Symbols used: S, sensitive; R, resistant

## Discussion

Most of the swine mycoplasma (acholeplasma) strains tested grew well in the BE medium, which had been shown earlier to suit the growth requirements of most bovine mycoplasma strains as well. *M. suipneumoniae* grew in all special media tested, but only under anaerobic conditions, whereas most other strains grew readily on both aerobic and anaerobic culturing. The growth of the acholeplasma strains was fast, that of mycoplasmas, especially of *M. suipneumoniae*, was much slower.

The finding that the mycoplasma (acholeplasma) counts of the cultures are related with the colour change caused by decomposition of glucose or arginine, seems to be of importance. The intensity of the yellow discoloration of glucose-containing broth cultures of *A. laidlawii* strains was directly related with the CFU for 5—6 days; in *A. granularum* cultures, the same phenomenon lasted only 4 days. The counts of *M. hyosynoviae* cultures were already fairly

low when the colour change became apparent. Shift of the pH by more than 0.5 grade step resulted in a marked decrease of the mycoplasma count of *M. suipneumoniae* cultures.

This correlation should be taken into consideration when first isolations, transfers or biochemicaı examinations of mycoplasma (acholeplasma) strains are made.

The main differentiating feature of mycoplasma and acholeplasma strains is cholesterol requirement. The methods proposed for its examination (EDWARD, 1971; RAZIN and TULLY, 1970) can be replaced for routine purposes by the much simpler tests of sensitivity to sodium polyanethol sulphonate and digitonin.

Important information for species differentiation can be obtained from biochemical and resistance tests, e.g., *A. granularum* can be differentiated from *A. laidlawii* by lack of aesculin hydrolysis and sensitivity to sodium chloride, Tween 80 and pH 5.5. The present findings are in good accordance with TULLY and RAZIN's (1968) observation that *A. laidlawii* A and B strains cannot be differentiated from one another on the basis of behaviour in biochemical tests, a slight difference being found only in sensitivity to pH 5.5.

The differentiation of mycoplasma strains is also possible by biochemical tests. *M. hyosynoviae* decomposes arginine, but does not break glucose, whereas *M. hyorhinis* behaves just oppositely. As the sugar-decomposing activity of the latter strain is often very slight, complementary tests for phosphatase activity and sensitivity to sodium azide and erythromicin are generally useful.

*M. suipneumoniae* behaves differently from both aforementioned species, especially in respect of growth ability, nutritional requirements, distinctive erythromycin sensitivity, decomposition of galactose and xylose and reduction of triphenyl tetrazolium chloride. As *M. hyorhinis* and *M. suipneumoniae* frequently occur in one and the same host and the former grows more readily in the special media than the latter, only an approximate indentification of already isolated cultures is possible by the mentioned tests. The differentiation of the two species in mixed cultures is, however, impossible, because they behave almost identically in the resistance tests. The fastly growing *M. hyorhinis* is less sensitive to erythromycin than *M. suipneumoniae*, whence the selection of the latter cannot be carried out by suppression of the former's growth with the antibiotic.

In summary, it is advised that with unknown, new mycoplasma isolates certain biochemical and resistance tests be performed before they are subjected to the extraordinarily work-consuming serological examinations. On the basis of the premilinary results, testing with fewer type sera is sufficient to confirm the preliminary classification of the isolate. The following tests are helpful in preliminary identification: cholesterol requirement, resistance to sodium polyanethol sulphonate and digitonin, decomposition of glucose, arginine,

galactose, xylose and aesculin, film and spot formation, phosphatase activity and resistance to sodium chloride, sodium azide, pH 5.5 and 9.5, Tween 80 and erythromycin.

## Summary

The properties of representative strains of various swine mycoplasma and acholeplasma species were compared by standard methods. The following biochemical properties were found to serve as important criteria of species differentiation: cholesterol requirement, sensitivity to sodium polyanethol sulphonate and digitonin, decomposition of glucose, arginine, aesculin, galactose and xylose, phosphatase activity, film and spot formation, and resistance to sodium chloride, sodium azide, pH 5.5 and 9.5, Tween 80 and erythromycin.

A correlation was demonstrated between changes of the pH of medium and the number of colony-forming units (CFU) in the cultures; this correlation should be taken into consideration at the propagation and transfer of mycoplasma (acholeplasma) strains.

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The address of the authors: Dr. László Stipkovits, Lea Varga, 1581 Budapest, Hungária krt. 21, Hungary; Dr. Dietrich Schimmel, Jena-Zwätzen, Naumburger Str. 96a, DDR.

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# INTERFERON INDUCTION BY DIFFERENT STRAINS OF INFECTIOUS BRONCHITIS VIRUS

By

B. LOMNICZI

Veterinary Medical Research Institute of the Hungarian Academy of Sciences
(Director: D. DERZSY), Budapest

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Infectious bronchitis virus (IBV) belongs to the *Coronavirus* group (ESTOLA, 1970) and is known to have several strains differing in antigenic composition and pathogenicity to chickens.

Little is known about its multiplication and other biological properties such as interferon (IF) induction. In our best knowledge only one report has been published on the IF production of an IBV strain (YUROV, 1967).

The present communication deals with the IF-inducing ability of various IBV strains and some characteristics of the IF production during multiplication of IBV.

## Material and Methods

*Viruses.* Strains Beaudette 222 (B222) and Massachusetts 41 (M41) of IBV were kindly provided by V. von Bülow, Bundesforschungsanstalt für Viruskrankheiten der Tiere, Tübingen; strains LED and PV were isolated from local epidemics in Hungary (LOMNICZI and STIPKOVITS, 1968). Some data concerning the pathogenicity of various strains are summarized in Table I.

**Table I**

Some biological properties of IBV strains

| IBV strains | Activity in | | | |
|---|---|---|---|---|
| | Chicken | Chicken embryo | | CEF |
| | | Passage | Survival, hours | |
| B222 | No symptoms | >200th | < 40 | CPE, growth |
| LED | Symptoms | 48th | > 48 | No CPE, no growth |
| M41 | Symptoms | 4th | > 96 | No CPE, no growth |

The origin of the other viruses such as Newcastle disease virus (NDV), Sindbis virus and Semliki Forest virus (SFV) has been described elsewhere (LOMNICZI, 1973).

*Propagation and titration of viruses.* These were carried out in 9-days old eggs as described earlier (ANON., 1963; LOMNICZI and STIPKOVITS, 1969; LOMNICZI, 1973).

*Cells.* Chicken embryo fibroblast cell (CEF) cultures were prepared according to LOMNICZI and BURKE (1970).

*IF production, IF titration and pretreatment of cells with IF* were done as described previously (LOMNICZI, 1973). IF titrations were performed in tube cultures by recording inhibition of cytopathic effects (CPE) of Sindbis virus. IF yields were expressed in international units (I. U.) established on the basis of comparison with Chick Research Standard A 62/4 sample, kindly provided by the Medical Research Council, London.

## Results

*IF production in chickens.* Six-week-old chickens were inoculated with doses of $10^7$ $ELD_{50}$/100 g body weight of IBV. Of three strains studied, only strain B222 induced measurable amounts of IF (20 I. U./ml) in the sera of chickens as tested 4 hours post-infection.

*IF production in eggs.* In embryonated eggs only strain B222, which killed the embryo rapidly, induced IF, while strain LED, which had undergone much fewer passages in chick embryo, failed to induce measurable amounts of IF (Fig. 1).

*IF induction in CEF.* Of the three strains studied only B222 induced IF in chick cells (Fig. 2). Appearance of IF coincided with that of CPE and attained maximum by 48 hours following infection. At that time about 90 per cent of cells showed CPE characteristic for IBV. Both strains LED and M41 had elicited neither IF induction nor CPE in CEF culture by the 96th hour after infection.

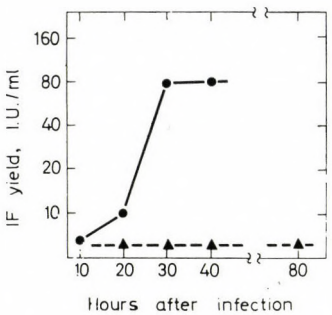


*Fig. 1.* Induction of IF with IBV strains in egg. B222 ●———; LED ▲– –

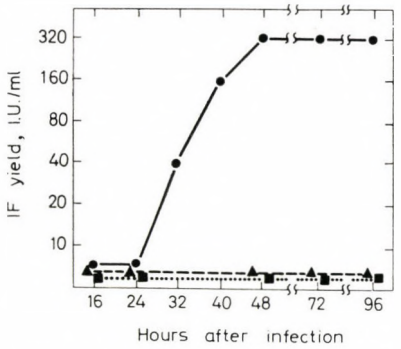


*Fig. 2.* Induction of IF with IBV strains in CEF cultures. B222 ●——; LED ▲– –; M41 ■------

*Attempts to render non-inducer strains to be inducers.* According to our experiences (Lomniczi, 1973, 1974) two methods, namely irradiation with ultraviolet light (UV) of the virus or IF-pretreatment of chick cells rendered both NDV and Aujeszky's disease virus good inducers. However, when strain LED was examined under the same conditions no IF could be detected (Table II).

**Table II**

Attempts to convert strain LED into an IF inducer

| Virus treatment | Cell treatment | IF yields | |
|---|---|---|---|
| | | 24 hours | 48 hours |
| None | none | < 10 | < 10 |
| UV | none | < 10 | < 10 |
| None | IF* | < 10 | < 10 |

* Cells were pretreated with 80 I.U. of IF for 18 hours.

*Conditions influencing IF induction by IBV.* Effects of certain treatments on IF induction by strain B222 are shown in Fig. 3 and Table III. IF induction

**Table III**

Effect of trypsin, heat and UV-irradiation on the IF-inducing ability of IBV

| Virus | | | IF yield | |
|---|---|---|---|---|
| Strain | Treatment | Titre $ELD_{50}$ | 40 hours | 56 hours |
| B222 | none | $10^{7}$ | 40 | 320 |
| B222 | trypsin, 60 min. | n.d. | 40 | 320 |
| B222 | UV, 4 min. | $10^{1.5}$ | < 10 | < 10 |
| B222 | 37 °C, 1 day | $10^{2.8}$ | 10 | 20 |

n.d. = not done

was unaffected by trypsin treatment of the virus, whereas it was significantly decreased by other treatments affecting the infectivity (exposure to heat and UV-irradiation) or reducing the multiplication capacity of the virus (IF-pretreatment of cells). Accordingly, CPE became less distinct under these conditions. The close relationship between virus multiplication and IF induction is reflected by the relative independence of IF yields at late stage of infection (56 hours) on the size of the inoculum (Fig. 4.) This suggests that at multiplicities of not higher than 1.0 some product during virus replication serves as IF inducer rather than the input virus itself.

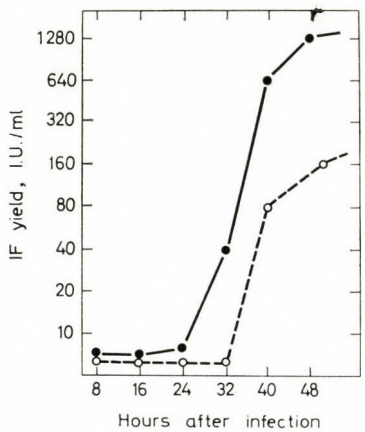


*Fig. 3.* Effect of IF-pretreatment on IF induction by strain B222. Normal cells ●——; IF-pretreated cells ○— — —

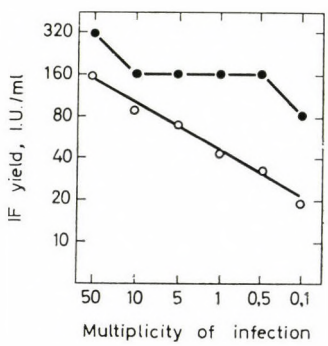


*Fig. 4.* Effect of virus dose on IF induction with B222. IF at 40 hours ○——; IF at 56 hours ●——

*Interference by IBV strains.* When CEF cultures infected with various IBV strains were superinfected with NDV, only B222 showed a significant interference activity in CEF (Table IV.)

**Table IV**

Interference of IBV strains with NDV

| Interfering strains | CPE and yield of NDV |
|---|---|
| None | 90% $3.2\times10^6$ PFU/ml |
| B222 | 0% $3.2\times10^5$ PFU/ml |
| LED | 50% $2.4\times10^6$ PFU/ml |
| PV | 80% $2.4\times10^6$ PFU/ml |

Superinfection with NDV was carried out 14 hours after infection with IBV.

## Discussion

The present studies have revealed that from different strains of IBV only B222 proved to be a good IF-inducer in various systems. In the chicken embryo and CEF culture B222 not only induced significant amount of IF, but also showed a higher virulence than strains LED or M41. In the egg maximal IF yield occurred when the embryo died and in CEF, when more than 75 per cent of cells showed CPE. Coincidence of higher virulence with better IF induction has been described in other virus-cell systems as well, namely during infection of western equine encephalitis virus (LOCKART, 1963), mengovirus (CAMPBELL and COLTER, 1967), Kilham rat virus (KILHAM *et al.*, 1968) and NDV (LOMNICZI, 1970, 1973).

Strains LED and M41 seem to be *ab ovo* unable to induce IF in contrast to NDV, another virus being noninducer in untreated state. However, NDV can be converted to be an inducer, suggesting that some product of the virus or of the replicative cycle may prevent the expression ot the IF-inducing ability in NDV-infected cells (SHEAFF *et al.*, 1972; LOMNICZI, 1973), in contrast to non-inducer strains of IBV.

IF induction by B222 is closely associated with virus replication, for IF production could be prevented by either inactivation of virus infectivity or inhibition of virus multiplication with IF. On the basis of these observations the type of IF induction by IBV is largely similar to that of SFV since the same treatments resulted in the same response as far as IF production is concerned (LOMNICZI, 1973). In SFV-infected CEF culture an IF treatment resulting in a 2000-fold decrease in virus yield caused more than 90 per cent inhibition of IF production (B. LOMNICZI, unpublished results). It has been shown that in the case of SFV, IF inhibits virus RNA synthesis (MÉCS et al., 1967) and RNA synthesis in turn is necessary to optimal IF induction (LOMNICZI and BURKE, 1970). Consequently, it is possible that in IBV-infected CEF cells it is also the virus RNA accumulating during virus replication which serves as IF inducer.

Strain B222, which was able to multiply in CEF to a limited extent, interfered with the growth of NDV, while other strains did not. This fact suggests that the latters have negligible activity in CEF. These findings are in accordance with those of BEARD (1967), who also observed that some viral activity was needed for IBV in interfering with NDV.

## Summary

Interferon- (IF) inducing ability and conditions of IF production by three strains of infectious bronchitis virus (IBV) were compared. Only strain Beaudette at high embryo passage showed significant IF induction in chickens, in eggs and in chick embryo cell culture. Maximal yield of IF in chick cells occurred at 48 hours when about 80 per cent of the cells showed destruction. Virus inactivation by heat or ultraviolet light, and suppression of virus growth by IF-pretreatment of cells, reduced IF production. IF production did not decrease significantly when the multiplicity of infection was reduced from 50 infectious units to 0.1, therefore, it has been concluded that molecules accumulating during virus replication and capable of inducing IF are more likely candidates for inducer than the RNA of the input virus. These conditions affected IF induction of Semliki Forest virus likewise, thus the IF inductions, by SFV and IBV are of similar types. Potentiating effects, such as UV-irradiation of the virus and pretreatment of the cell with IF, failed to convert two non-inducing strains of IBV into IF inducers.

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Address of the author: Dr. Béla LOMNICZI, 1581, Budapest, Pf. 18. Hungary.

# BIBLIOGRAPHIA

GREENOUGH, P. R., MACCALLUM, F. J. and WEAVER, A. D.: *Lameness in cattle.* Oliver and Boyd, Edinburgh, 1972. Pp. 478.

The volume is divided into three main parts and a total of 43 chapters.

The first part (eight chapters) discusses the forms of lameness in cattle in general, with regard to economic importance. This is followed by a description of the functional anatomy of the extremities. The methods of sedative treatment as well as local and general anaesthesia are also outlined under this heading. Fixation and methods of examination of the extremities are described in great detail. The preparation of roentgenograms is demonstrated and instructions are given for their evaluation.

The second part (eleven chapters) deals with the diseases of the foot. The richly illustrated introductory chapter gives a detailed anatomical description of the region. The arteriographic and venographic illustrations of the blood supply of the foot deserve special mention.

The subsequent chapter deals with the diseases of the skin, horny part and corium of the foot in great detail. Communication of the names of diseases in several languages, including Chinese, in this and other chapters of the book greatly facilitates orientation in the literature. The chapters on ulcer of the sole and purulent processes of deep foot tissues are very instructive and well illustrated. The last chapter describes the modes of care and surgical treatment of the foot (partial amputation, resection of foot joint and of deep and superficial flexor tendons).

The third, most comprehensive part, consisting of 23 chapters, is devoted to diseases of the legs and thighs. A separate chapter deals with the diseases of the different anatomical structures, such as joints, tendons, bones, muscles and nerves. The most outstanding part of this chapter is the description of joint diseases, completed by excellent illustration.

It is a great merit of the authors that, apart from independent diseases of the extremities, they deal with secondary affections resulting from infectious diseases, and with extremity lesions related to dietary error.

The list of round 800 references can well be regarded as a complete bibliography of the topic. A detailed Subject Index facilitates orientation in the book.

Although the value of the illustration material has already been referred to in this review, it should be pointed out once again. The Figures, 252 black-and-white photos and 24 colour plates, distinguish themselves in addition to a great scientific value by a rare typographical perfection.

Such a detailed discussion of extremity diseases in cattle is unique of its kind in present-day veterinary literature. Although due consideration is given to the theoretical aspects of all topics, the main purpose of the book is clearly to satisfy the demands of practitioners and clinicians. The book is useful to all veterinarians, but cattle specialists in particular can rely on it as on an indispensable fundamental work.

Dr. László TAMÁS

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РЕЗЮМЕ

## ЧУВСТВИТЕЛЬНОСТЬ К ТУБЕРКУЛИНУ СЕНСИБИЛЬНЫХ ЛИМФОЦИТОВ

Ш. ТУБОЙ и Т. САНТО

Изучалась чувствительность лимфоцитов морских свинок, привитых живыми культурами разных штаммов **Mycobacterium bovis, M. avium, M. ulcerans** и **M. xenopei**, к антигенам разных микобактерий. В качестве антигена употреблялись препараты туберкулина **PPD**, очищенный антиген обозначения Ps и проверена их активность в провоцировании «бласт-трансформаций».

Сравнив индуктивный эффект бычьего и птичьего PPD, обнаружено, что в сенсибилизированных **M. bovis** лимфоцитах их показатели были близки друг к другу. Зато имелась существенная разница в этих показателях, если лимфоциты сенсибилизировались **M. avium**. Не было разницы в действии на лимфоциты упомянутых антигенов, если экспериментальных животных привили **M. ulcerans** и **M. xenopei** соответственно.

Антиген **Ps**, полученный из штамма **M. bovis** $AN_5$, вызывал бласт-трансформацию только в группе животных, привитых **M. bovis**. Лимфоциты других групп животных на этот антиген не реагировали.

## К ЭПИЗООТОЛОГИИ И КЛИНИКЕ ВИРУСНОЙ ПАНЛЕЙКОПЕНИИ КОШЕК

ХОРВАТ З., ПАПП Л. и БАРТА А.

На основании тщательных наблюдений на 1770 кошках изучались эпизоотология и клиника вирусной панлейкопении (инфекционного гастро-энтерита) кошек. Обособлены разные клинические формы болезни, разработан эффективный способ терапии и определена динамика обнаруживаемости болезни по отдельным порам года и возрастным группам. Проведены эксперименты по изоляции вируса и получению вакцины.

За десятилетие (1962—1972) среди 7944 кошек клиники 1770 (22,3%) болело панлейкопенией. Вспышки болезни наблюдались главным образом весной и осенью. Чаще болели кошки возраста 7—12 месяцев (38,8%).

Среди подострой, острой и хронической форм болезни чаще всего регистрируется острая. Для нее характерны высокая температура (40,4—42,0° С), повторные рвоты, тяжелый эксикоз и лейкопения (500—500 лейкоцитов в мм³). У одной части больных наблюдался некроз слизистой языка в виде 3—4 мм-й полосы (маргинальный абсцесс). Для хронической формы болезни характерны упорный кишечный катар, анемия и кахектическое состояние.

В терапии особое значение имеет внутривенное введение крови кошек старшего возраста или иммунизированных животных, введение жидкости (дегидрация) и для профилактирования вторичных бактериальных инфекций введение через рот неомицина порошка и хлорамфеникола инъекции.

Кроме тяжелой морбидности болезнь характеризуется высокой летальностью.

Вирус изолировался из разных органов 47 павших кошек. Из органов 6 животных удалось изолировать вирус панлейкопении во вторичной кошачьей почечной клеточной культуре.

Искусственное заражение вирусом показало, что инфекционный ларинго-энтерит и вирусная панлейкопения кошек это одна и та же болезнь.

При помощи вируса, культивировавшегося на вторичной кошачьей почечной клеточной культуре, получена обработанная формалином вакцина, не уступающая в эффективности вакцине «фелидовак» фирмы Берингверке.

## ВИДЫ ПАРАМФИСТОМУМОВ КРУПНОГО РОГАТОГО СКОТА И ОВЕЦ В ВЕНГРИИ

О. ШЕИ

Описываются три вида рода **Paramphistomum** (**P. daubneyi Dinnik, 1962, P. ichikawai Fukui, 1922, P. leydeni Näsmark, 1937**) из крупного рогатого скота и овец Венгрии. Среди них **P. daubneyi** обнаруживался только в крупном рогатом скоте, **P. ichikawai** и **P. leydeni** главным образом в овцах и реже в крупном рогатом скоте.

На основании специфичности промежуточного хозяина и морфологических свойств автором доказано, что раньше описанный из Венгрии вид **P. microbothrium** на самом деле является **P. daubneyi**.

Дается детальный анализ сходных признаков **P. (liorchis) scotiae** и **P. leydeni**.

## СЕРОЛОГИЧЕСКИЕ ИССЛЕДОВАНИЯ НА ОБНАРУЖЕНИЕ ВИРУСОВ, ВЫЗЫВАЮЩИХ ЗАБОЛЕВАНИЯ ДЫХАТЕЛЬНЫХ ПУТЕЙ И ПИЩЕВАРИТЕЛЬНОГО ТРАКТА КРУПНОГО РОГАТОГО СКОТА В ГОСУДАРСТВЕННЫХ ХОЗЯЙСТВАХ КОМИТАТА ФЕЙЕР

Э. МОЧАРИ, Э. ШАГИ, Ю. КИШШ,
Э. ФЮЛЕШИ и Л. ОВАРИ

Проводились серологические исследования в 14 государственных хозяйствах комитата (области) Фейер на выявление нейтрализирующих антител против вируса вирусного поноса (ВП), инфекционного бронхо-ринита (ИБР) и аденовирусов, дальше, комплемент связывающих антител против хламидий. Среди 1988 проб крови перечисленные антитела обнаружены в пробах крови из отдельных хозяйств в следующем соотношении:

| | | | |
|---|---|---|---|
| вирус ИБР | 0—52% | в среднем | 14,8% |
| вирус ВП | 7—73% | в среднем | 33,2% |
| аденовирус | 34—86% | в среднем | 66,2% |
| хламидия | 0—32% | в среднем | 8,2% |

Сравнивается результат серологических исследований проб крови из клинически здоровых и больных респираторными и энтеральными болезнями поголовьев. Авторы приходят к заключению, согласно которому в поголовьях с клиникой и экономическим ущербом антитела против ВП и аденовирусов обнаруживаются в большем проценте животных, чем в таковых клинически здоровых поголовьев. Такой разницы в обнаруживаемости антител против ИБР и хламидии не обнаружено.

На основании сказанного можно прийти к выводу, что аденовирусы и ВП играют более важную роль в этиологии вирусных заболеваний дыхательных путей и пищеварительного тракта среди крупного рогатого скота государственных хозяйств комитата Фейер, чем вирус ИБР и хламидии.

## ЗАБОЛЕВАЕМОСТЬ МОЛОДЫХ ГУСЕЙ ВЬЕТНАМА

ХАН Н. В.

В последние годы зарегистрированы вспышки заболеваний, которые на месте диагносцировали как чуму гусей. По своей эпизоотологии, клинике, патологоанатомической картине и патогистологическим изменениям они очень похожи на заболевания, именуемые в Венгрии инфлюэнцой гусей.

В Вьетнаме болеют обычно гусята 1—3-недельного возраста и смертность в первые годы достигает 60—90%. Описываются клинические признаки, патологоанатомические и патогистологические изменения болезни молодых гусей Вьетнама. В распространении болезни в Вьетнаме важнейшую роль играли не инкубационные станции, а очень живое перемещение населения и гусей, разных инфицированных средств ухода, являющихся следствием военных событий.

Из органов естественно заразившихся вьетнамских гусей автором изолирован вирусный штамм (обозначения V). Вьетнамский вирусный штамм V и венгерский вирусный штамм B размножаются в гистологической культуре фибробластов гусиного зародыша, но без оценимого цитопатогенного эффекта. Зародыш утки Cairina moschata восприимчив к вирусным штаммам V и B. Оба штамма резистентны к воздействию хлороформом, pH 3, температурой, 0,25% трипсина, 0,2% натрия додецилсульфата и 0,1% сапонина. После пассажа через гусиный зародыш вирулентность вируса V ослабела.

Пробы нейтрализации и перекрестной защиты сыворотки показали, что структура вьетнамского вирусного штамма V, наичаще обнаруживаемого в Венгрии штамма B и изолированного в Голландии вируса не отличаются друг от друга.

## ПОПУЛЯЦИОННО-ГЕНЕТИЧЕСКОЕ ИЗУЧЕНИЕ ТРАНСФЕРИНОВЫХ ВАРИАНТОВ В НЕКОТОРЫХ ПОПУЛЯЦИЯХ ВЕНГЕРСКОГО СКОТА

Г. ШООШ, Э. ГИППЕРТ и Г. ЧОНТОШ

В разных популяциях венгерского скота изучались геновая частота сывороточного трансферина и сегрегация аллелей среди 18 816 голов животных, служащих для 9166 случаев подбора. Обнаружено 6 трансфериновых аллелей. При спаривании гомозиготных коров и гетерозиготных быков среди телят обнаружено математически достоверное отсутствие гетерозиготных особей, то есть доказан т. наз. эффект. "**unlike-homozygous-mother effect**" (**maternal-foetal incompatibility**). Кроме этого при анализе по Каттаб и сотр. (1964) обнаружено не мало отклонений в сегрегации отдельных фенотипов аллелей. Данные являются подтверждением обнаруженного другими авторами факта, что полиморфизм трансферинов крупного рогатого скота не является уравновешенным полиморфизмом; считается вероятным, что он стоит под воздействием пока нерасшифрованных иммунологических или физиологических факторов.

## ДАННЫЕ К ДИПЛОСТОМОЗУ МАЛЬКОВ ТОЛСТОЛОБИКА

К. МОЛНАР

Среди 3-недельных мальков толстолобика (**Ctenopharyngodon idella**), выращивающихся в пруде, наблюдалась 90%-ная гибель. Причиной гибели мальков длины 1,7—2,5 см явились мигрирующие церкарии **Diplostomum spathaceum** В тяжело больных животных обнаружено 20—25 осевших личинок в чечевице глаза и 10—15 мигрирующих таковых. Мигрированием церкарий вызывались обнаруживаемые пустым глазом кровоизлияния.

Попавшие в орбитальную полость или глазное яблоко церкарии окружает, как правило, пролиферативная ткань.

## ДАННЫЕ К ГЕМОГРАММЕ БУЙВОЛА И ВЕРБЛЮДА

Й. БОКОРИ

Приводятся данные исследований крови (количество эритроцитов, белых кровяных телец, гемоглобина, гематокрита, качественной кровяной картины, РОЭ) 47 здоровых и страдающих разными болезнями взрослых буйволов и буйволят, а также 35 взрослых верблюдов. Сопоставляя немногочисленные литературные данные со своими, автор указывает на то, что среднее количество эритроцитов у больных животных (взрослые буйволы — 4,5 млн.; буйволята — 5,5 млн.) было меньшим, чем у здоровых животных (5,8 и 6,5 млн. соответственно). Количество белых кровяных телец у больных животных повысилось приблизительно на 50% (11 700 и 12 400 соответственно). В показателе гемоглобина и гематокрита не было существенной разницы между здоровыми и больными животными. В кровяной картине больных буйволов наблюдался сдвиг влево (66 и 57%-ная нейтро-

филия соответственно). РОЭ у больных животных, особенно в первые два часа, была быстрой. Средний диаметр эритроцитов равнялся 5,1 микронам.

В 1 мм³ крови верблюдов насчитывалось в среднем 5,5 млн. эритроцитов, 13 200 белых кровяных телец, обнаружено 11,8 г% гемоглобина и 29% гематокрита. Кровяная картина была то слабо лимфоцитарной, то нейтрофильной. Темп РОЭ у верблюдов был очень медленным и оседание закончилось только после 72—96 часов; с 24-ого часа оно было часто двухфазным.

## ЛИХОРАДОЧНАЯ, ОБЩАЯ ФОРМА ИНФЕКЦИОННОГО РИНОТРАХЕИТА СРЕДИ ТЕЛЯТ

А. БАРТА, Я. КИШАРИ и Ш. БЕЛАК

В поголовьях крупного рогатого скота крупных хозяйств, особенно среди телят 1—6-месячного возраста, реже среди молодняка и племенных животных, часто регистрировалось тяжелое заболевание при наличии клиники со стороны дыхательных путей, наносящее большой экономический ущерб. Температура больных телят повысилась до 41—42° С, животные были угнетенными, у них наблюдался отказ от корма, истечение слизи из носовой полости и пенистое слюноотделение, иногда болезненный сухой кашель. После острых признаков часть животных выздоровела, у другой части развилась хроническая серозно-гнойная пневмония. Падеж иногда достигал 20%. У взрослых животных бесхарактерное, острое, лихорадочное заболевание дыхательных путей проходило доброкачественно, без возникновения сопутствующей пневмонии. Из больных телят в тканевых культурах удалось изолировать вирус IBR. Данное заболевание по эпизоотологии и клинике отличается от доселе известных 5 форм болезни (заболеваемость верхних дыхательных путей, вирусное воспаление влагалища, энкефалит, конъюнктивит, аборт), вызываемой вирусом IBP; поэтому это общее лихорадочное заболевание дыхательных путей можно считать 6-ой формой болезни, вызываемой вирусами IBR.

Этой формой болезни в Венгрии наносится больше ущерба, чем всеми остальными формами вместе.

## ЭЛЕКТРОННО-МИКРОСКОПИЧЕСКОЕ ИЗУЧЕНИЕ СТРУКТУРЫ ЖЕЛЕЗИСТОГО ЖЕЛУДКА КУРЫ

И. ХОРВАТ

Изучены все типы эпителиальных клеток железистого желудка куры.

Эпителиальные клетки просвета желудка и таковые желез проприи по строению не отличались от поверхностных клеток млекопитающих, выделяющих муцин. Поскольку разницы в строении эпителиальных клеток железистого желудка и клеток крипт не обнаружено, автор сомневается в правильности отдифференциации последних при помощи термина «железы проприи».

Большую часть стенки железистого желудка составляют «железы субмукозы», организованные в множественные кругловатые или неправильной формы дольки. Тубулезные железистые желудочки, подобно низшим позвоночным, выстланы так называемыми «оксинтико-пептическими» клетками. Их ультраструктура является характерной смесью таковой главных и обкладочных клеток, что является следствием двойной функции единой клетки. Клетки выделяют содержимое беспрерывно. В условиях кормления вволю интенсивность выделения зависит главным образом от поры дня.

Между зрелыми «оксинтико-пептическими клетками» имеются недифференцированные и «аргентафинные» клетки.

## ДАННЫЕ К «ОКТОМИТОЗУ» (СПИРОНУКЛЕОЗУ) ЦИПРИНИД И АКВАРИЙНЫХ РЫБ

К. МОЛНАР

На основании данных изучения нескольких случаев автор приходит к заключению, согласно которому жгутиковые семейства **Diplomastiginae** для ципринид и аквариийных рыб такого же патологического значения, какое они представляют при так называемом октомитозе построгов.

Виды, включенные автором в род Spironucleus, могут вызывать гибель амура, усача и лестнички.

У амура паразиты вызывают энтерит, асцит и общую водянку. У усача наблюдались энтерит, некроз печени и образование серозного выпота в брюшной полости; у лестнички энтерит, далее кроветеки и некроз по всему телу, но особенно на голове. Характерностью гибели этих рыб явилось, что эти типичные кишечные паразиты в большом количестве обнаруживались в печени усача и в крови амура и лестнички.

## ИЗУЧЕНИЕ ТЕМПЕРАТУРНОЙ ЧУВСТВИТЕЛЬНОСТИ ГЕМОЛИЗИНА, СВЯЗАННОГО С КЛЕТКОЙ ESCHERICHIA COLI

Ф. МУРАНИ и Ш. ЮХАС

Авторами изучалась температурная чувствительность гемолизина, связанного с клеткой Escherichiacoli. Они обнаружили, что от термообработки без существенного повреждения микробного числа гемолизирующий эффект прекращается. В пределах 42—50° С как начальные показатели понижения титра, так и срок полного его исчезновения при изображении функционального взаимоотношения с температурой показывает экспоненциальную регрессию. В трансформированной системе показатели начального понижения титра и его полного исчезновения изменяются параллельно.

## ЭКСПЕРИМЕНТЫ ПО ВАКЦИНАЦИИ ПРОТИВ ВИРУСНОГО ПОНОСА СВИНЕЙ

### I. ВЗАИМОСВЯЗЬ МЕЖДУ ВАКЦИНИРОВАНИЕМ СВИНОМАТОК И КОЛОСТРАЛЬНЫМ ИММУНИТЕТОМ ПОРОСЯТ

Л. ЧОНТОШ, Т. СЕНТ-ИВАНИ и Я. БЕНЕДА

Проводились эксперименты вакцинирования вирусом поноса свиней (transmissive gastro-enteritis, TGE) обозначения СКр, пассированного 15 и 27 раз в свиной почечной и свиной щитовидножелезистой клеточной культуре соответственно. В двух стадах вакцинировано всего 77 супоросных свиноматок 3—10 недель до опороса. Одна часть свиноматок вакцинировалась один раз, другая — два раза с промежутком времени в 2, 3 и 6 недель соответственно. Вируссодержащая жидкость вводилась в носовую полость.

Вакцинирование свиноматки переносили бессимптомно. Выделение вируса наблюдалось только у одной свиноматки, привитой непосредственно перед опоросом. В крови поросят этой свиноматки появились в высокой концентрации вирус нейтрализирующие антитела. Вакцинный вирус оказался безвредным в отношении поросят.

После вакцинации и потом после опороса пробой нейтрализации регистрировали появление и изменение концентрации антител в крови и молозиве. Наиболее удобным оказалось вакцинирование два раза с промежутком времени в 6 недель. От вакцинаций с промежутком в две недели в крови и молозиве свиноматок обнаружен высокий уровень антител.

Эффективность вакцины контролировали заражением поросят. Обнаружено, что на неблагополучном месте защиту поросят можно обеспечить только в том случае, если они бесперебойно будут получать молозиво и молоко, содержащее антитела. Если уровень антител в молоке понизился, или поросят отбили от матки после высосания молозива, они легко заражались, хотя их кровь всегда содержала антитела. Если поросята бесперебойно снабжались антитела содержащим молоком, то они не болели на неблагополучном месте и под защитой антител приобретали иммунитет.

## ЭКСПЕРИМЕНТЫ ПО ВАКЦИНАЦИИ ПРОТИВ ВИРУСНОГО ПОНОСА СВИНЕЙ

### II. ПЕРСИСТИРОВАНИЕ ТИТРОВ ПОКАЗАТЕЛЕЙ И ИХ ИЗМЕНЕНИЕ ПОСЛЕ ПОВТОРНОЙ ВАКЦИНАЦИИ

Я. БЕНЕДА, Э. МОЧАРИ и Е. ШАГИ

Продолжая начатые раньше эксперименты по вакцинации против вирусного поноса свиней (transmissive gastro-enteritis, TGE) у свиноматок, привитых полгода тому назад вакциной с живым вирусом, изучали персистирование титров антител и их изменение после повторной вакцинации.

Изучая персистирование показателей титра, обнаружили, что средний титр на 2,27 log выше такового у свиноматок, привитых полгода тому назад. Он же у животных, дважды привитых с промежутком времени в две недели, не изменился; у дважды привитых животных с промежутком времени в 3 и 6 недель титер понизился на 0,89 log 2 и 3,25 log 2 соответственно. Понижение высоких титров всегда было более существенным, чем низких.

Показатели титра у повторно привитых свиноматок зависели от уровня титра антител в момент их второй прививки. Низкие показатели титра поднялись более существенно, чем высокие. От повторной ревакцинации не получено существенно лучшего результата, чем от разовой таковой.

На основании данных авторы приходят к заключению, согласно которому персистирование показателей титра наиболее выразительно после двухкратной вакцинации с промежутком времени в две недели, то есть разовым повторением вакцинации полгода спустя уровень антител у свиноматок формируется наиболее благоприятно.

## ИЗУЧЕНИЕ КОКЦИДИОЗОВ КОШКИ

Л. ПЕЛЛЕРДИ

Дается описание нового вида кокцидии кошки (Felis catus) Isospora novocati sp. n., напоминающего немножко собачий вид Isospora rivolta. Оба вида можно легко отдифференцировать друг от друга. Кругловатые или короткие эллипсоидные ооцисты промера 24—30 $\mu$м (круглые формы), или 22—30 x 29—27 $\mu$м. Срок спорулирования при комнатной температуре равняется 1—2 дням, препатентный период — 6 дням. Собак не удалось инвазировать его спорулированными ооцистами. Массивная инвазия вызывала у кошек переходный понос.

## ИЗУЧЕНИЕ ПРОДУКЦИИ ЖЕЛУДОЧНОГО СОКА У ПОРОСЯТ В СВЯЗИ С РАННЕЙ ОТБИВКОЙ

Ф. КУТАШ и Й. САБО

Проведено сравнительное изучение продукции желудочного сока у отбитых и подсосных поросят с 1-ой до 4-й недели жизни. Желудочная слизистая оболочка стимулировалась подкожным введением гистамина, секреторный же ответ изучался проверкой pH желудочного сока. Большинство подсосных поросят было ахлорогидрозным или гипохлорогидрозным: выделения соляной кислоты после введения гистамина или совсем не наблюдали, или обнаружено совсем слабое ее выделение в желудочном соке. Зато большинство отбитых в 1-недельном возрасте поросят после инъекции гистамина выделяло с желудочным соком соляную кислоту.

На основании полученных данных можно заключить, что срок ахлорогидрии существенно короче в случае ранней отбивки поросят, чем у подсосных животных. Обнаружено, что выделение соляной кислоты наступает скоро после отбивки. Раннее появление желудочной кислоты благотворно сказывается на желудочном протеолизе, кроме этого она может профилактировать пероральное заражение.

## СЕРОЛОГИЧЕСКОЕ ИЗУЧЕНИЕ БЫЧЬИХ МИКОПЛАЗМ И АХОЛЕПЛАЗМ

Л. ШТИПКОВИЧ и Л. ВАРГА

Референтные штаммы микоплазм и ахолеплазм бычьего происхождения изучались в пробах торможения роста и метаболизма. Доказано наличие двухсторонней перекрестной реакции между серогруппами 7 PG 50 (Leach) и L B 144P/Al-Aubaidi в пробе метаболизма и односторонней таковой в пробе торможения роста. Метаболическая активность **M. agalactiae subsp. agalactiae (PG 2)** тормозилась антисывороткой **M. agalactiae subsp. bovis (Donetta).**

Серогруппы, обозначенные буквами H, I и K (Al-Aubaidi), показывали статистически достоверные двухсторонние перекрестные реакции в обеих пробах с видами **M. gateae, M. gallinarum** и **A. axanthum,** подтверждая правильность наблюдений Эрно, Юрманова и Лич (1973).

## ТОНКАЯ СТРУКТУРА СПОРОЗОИТОВ ВИДОВ LANKESTERELLA, ПАРАЗИТИРУЮЩИХ В RANA PIPIENS

Г. ХЕЛЛЕР

Тонкая структура видов рода **Lankesterella** почти идентичная. Мембрановидные (сосиско образные) органеллы кажутся особенно характерными для всех видов. Пелликула при движении образует невысокие складки. Форма этих складок или «волн» сравнивается со складками спорозоитов **Eimeria stiedai.** Паразитофорная вакуоль наполнена малыми пузырьками, оторвавшимися от мембраны хозяинной клетки.

## ИММУНОЛОГИЧЕСКОЕ ИЗУЧЕНИЕ ИНФЕКЦИОННОГО ГАСТРОЭНТЕРИТА СВИНЬИ

Я. БЕНЕДА, Э. МОЧАРИ и ДЬ. ГАЙДАЧ

Во время вспышки инфекционного гастроэнтерита свиней (transmissible gastroenteritis) изучалась корреляция между понижением и прекращением заболеваемости и смертности от этой болезни с одной стороны и повышением антител в молозиве и молоке маток, с другой. Образцы крови и молока брались от тех свиноматок, которые поросились к 12-у и 13-у дням после наступления болезни. Образцы крови, полученные в день опороса и на 7 дней позже, и образцы молока, полученные в день опороса и после 3, 5 и 7 дней, изучались на вирус нейтрализирующие антитела. Титры антител были отрицательно пропорциональны заболеваемости и смертности поросят в момент вспышки болезни. На основании полученных данных можно прийти к заключению, согласно которому заболеваемость и гибель среди поросят профилактируется только при соотношении титра антител в молозиве и молоке 1 : 8.

## К ЦИКЛУ РАЗВИТИЯ EIMERIA LEPORIS И ЕЕ ПАТОГЕННОСТИ ДЛЯ ЗАЙЦА

Л. ПЕЛЛЕРДИ, М. ХЁНИХ и Л. ШУГАР

Изучение тушек спонтанно инвазированных зайцев и двух искусственно зараженных молодых зайцев (**Lepus eupaeus**) показало, что массивная или повторные инвазии спорулированными ооцистами **Eimeria leporis** могут быть причиной летального кокцидиоза. Дается новое описание морфологии ооцист **E. leporis,** детальное описание эндогенных стадий развития и выяснен препатентный период развития, который равняется 7 дням. Два молодых зайца, страдающих слабым кокцидиозом **E. leporis,** от заражения через рот 2 миллионами спорулированных ооцист смертельно заболели. На основании этого авторы заключают, что к **E. leporis** вырабатывается только слабый иммунитет. Слабая иммуногенность вида может быть причиной его широкого распространения.

## ЭКСПЕРИМЕНТАЛЬНЫЙ ПОНОС У ДНЕВНЫХ КРОЛЬЧАТ, ВЫЗВАННЫЙ ВВЕДЕНИЕМ ЧЕРЕЗ РОТ ЭНТЕРОПАТОГЕННОГО ДЛЯ СВИНЬИ ТЕПЛО-ЛАБИЛЬНОГО ЭНТЕРОТОКСИНА E. COLI

Ф. КУТАШ, Ф. ВЕТЕШИ и Г. ШЕМЬЕН

Изучались патофизиологические изменения у подсосных крольчат, вызванные энтеротоксином E. coli, и сравнивались с ответными реакциями контрольных животных, обработанных полным клеточным лизатом не энтеротоксических штаммов.

Доказано, что тепло-лабильный токсин, продуцируемый энтеропатогенным штаммом E. coli свиного происхождения, вызывает понос с характерными клиническими симптомами и патологоанатомическими изменениями; полный клеточный лизат не энтеротоксических штаммов E. coli этих симптомов не вызывает.

Энтеротоксином вызванные изменения очень напоминали таковые при естественном мукоидном энтерите кроликов и колибациллезном поносе поросят: усиливается секреция в просвет кишечника, наступает понос, тяжелая дегидрация, метаболический ацидоз и уремия.

Изучены количество и состав жидкости, накопившейся в кишечнике. По сравнению с плазмой хозяина она богата бикарбонатом и изоосмотична с ней.

Подсосные крольчатакажутся удобной моделью для изучения эффекта энтеротоксина E coli.

## СРАВНИТЕЛЬНОЕ ИЗУЧЕНИЕ СВИНЫХ ВИДОВ РОДА MYCOPLASMA

Л. ШТИПКОВИЧ, Д. ШИММЕЛ и Л. ВАРГА

Стандартными методами изучены свойства репрезентативных свиных видов рода **Mycoplasma** и **Acholeplasma**. Для дифференциации видов удобны следующие обнаруженные признаки: потребность в холестероле, чувствительность к натрию полианетола, сульфонату и дигитонину, расщепление глюкозы, аргинина, эскулина, галактозы и ксилозы, фосфатазная активность, образование пленки и пятна; резистентность к хлориду натрия, азиду натрия, pH 5,5 и 9,5, Твин 80 и эритромицину.

Обнаружена взаимосвязь между pH питательной среды и количеством колонио формирующих единиц (**colony-forming units**) в культурах; это соотношение нужим учитывать при культивировании и пересеве микоплазмозных (ахолеплазмозных) штаммов.

## СПОСОБНОСТЬ ИНДУКЦИИ ИНТЕРФЕРОНА РАЗНЫМИ ШТАММАМИ ВИРУСА ИНФЕКЦИОННОГО БРОНХИТА

Б. ЛОМНИЦИ

Сравнивались способность индукции интерферона и условия его образования тремя штаммами вируса инфекционного бронхита. Только штамм Бодет после большого количества пассажей через куриный зародыш индуцировал в цыплятах, в яйцах и клеточной культуре из куриного зародыша образование интерферона. Максимальное количество интерферона в цыплячьих клетках обнаруживалось к 48 часам, когда примерно 80% клеток показывало деструкцию. Инактивирование вируса температурой и ультрафиолетовыми лучами и торможение размножения вируса путем предварительной обработки клеток интерфероном снижают продукцию интерферона. Продукция интерферона не понизилась статистически достоверно, если интенсивность заражения снизили с 50 инфекционных единиц до 0,1, поэтому предполагается, что источником индукции являются молекулы, накопляющиеся при размножении вируса и способные продуцировать интерферон, а не РНК выделяющегося вируса. Эти же условия действуют на индукцию интерферона вирусом Семлики Форест. Таким образом, индукции интерферона вирусами Семлики Форест и инфекционного бронхита являются подобного типа. Потенцирующим воздействием, а именно облучением вируса ультрафиолетовыми лучами или предварительной обработкой клеток интерфероном, не удалось преобразовать двух штаммов вируса инфекционного бронхита, не индуцирующих образование интерферона в индуцирующие таковые.

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# VISCERAL LARVA MIGRANS OF *NEOASCARIS VITULORUM* IN LABORATORY ANIMALS

By

O. A. ATALLAH, I. MOSSALAM and M. S. ABDEL-RAHMAN

Pathology, Microbiology and Parasitology Dept. Faculty of Vet. Med. El Zagazig and Pathology and Parasitology Department Faculty of Vet. Med. Cairo University

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The reactions to parasites of man in abnormal hosts have been extensively studied. The reverse situation, in which man himself is the abnormal host for parasites of other animals, has received considerably less attention.

A few cases were reported by TAKATA (1951), and MENDHEIM et al. (1952) in which animal ascarids have infected humans and developed to sexual maturity in much the same manner as in their normal hosts. Due to its rarity, the importance of this type of infection is relatively small, yet, it cannot be overlooked. On the ground of a study of cutaneous larva migrans BEAVER et al. (1952) believe that various nematode larvae behave similarly both in man and animals.

More important phenomena of animal ascarid infection in humans involve the type called visceral larva migrans by BEAVER et al. (1952). BEAVER (1954), SMITH and BEAVER (1953) and BEAVER (1956) suggest that this condition may exist more frequently than is commonly presumed, especially in children. DENT et al. (1956) state that the close association of young children with dogs and cats establishes an ideal set of circumstances under which infective ova may be ingested. The infective eggs of *Neoascaris vitulorum* may cause the same syndrome when swallowed by the farmers living in close contact with the farm animals, especially cattles and buffaloes.

The present authors have undertaken a fundamental study on the progress of histopathological changes in the laboratory animals experimentally infected with *N. vitulorum*.

## Materials and Methods

Rabbits, guinea pigs, rats and chickens of 40, 75, 20 and 7-day-old-respectively, were used in this work. Five individuals from each species were experimentally infected with about 5000 infective eggs of *Neoascaris vitulorum*. One animal of each group was slaughtered 1, 2, 3, 7 and 10 weeks after infection. The methods of obtaining eggs, infection and processing tissues are the same as described by MOSSALAM et al. (1971 and 1972).

## Results

*Liver*

Rat: Liver of the infected rats showed some lymphocytic infiltration around the bile ducts.

Guinea-pig: The larvae were present among hepatic cells (Figs 1, 2). They were apparently alive or degenerated and surrounded by eosinophils,

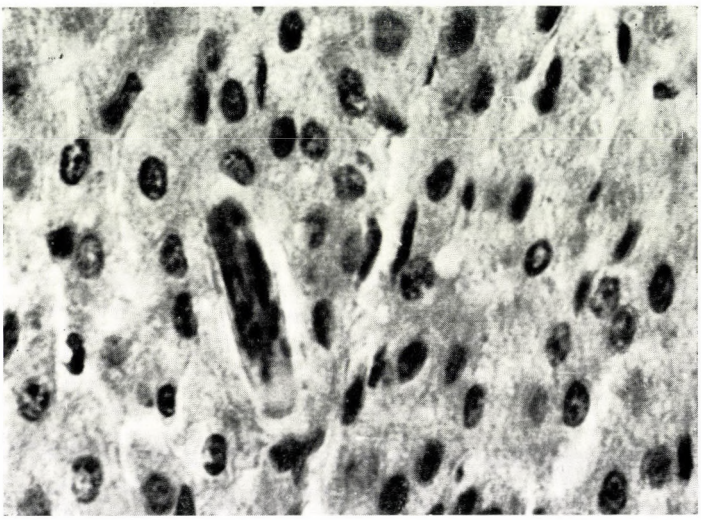

*Fig. 1.* Larvae among hepatic cells without inflammatory reaction in liver of guinea pig 7 days after infection. Haem.–eos. ×1000

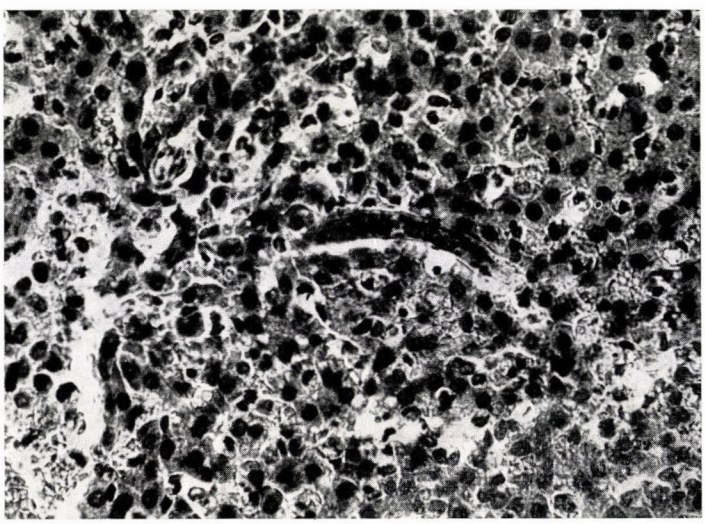

*Fig. 2.* Larva among hepatic cells of guinea pig 21 days after infection. Haem.–eos. ×400

neutrophils, fibroblasts and cellular debris 3 weeks after infection. Patches of variable size showing coagulative necrosis of hepatic cells and subcapsular haemorrhages were frequently seen. Few eosinophils infiltrated the interstitial tissue and some hepatic cells were dissociated. Portal tracts presented numerous eosinophils inside the congested portal veins thickened hyalinized wall of hepatic arterioles, infiltration with lymphocytes and eosinophils and prolifer-

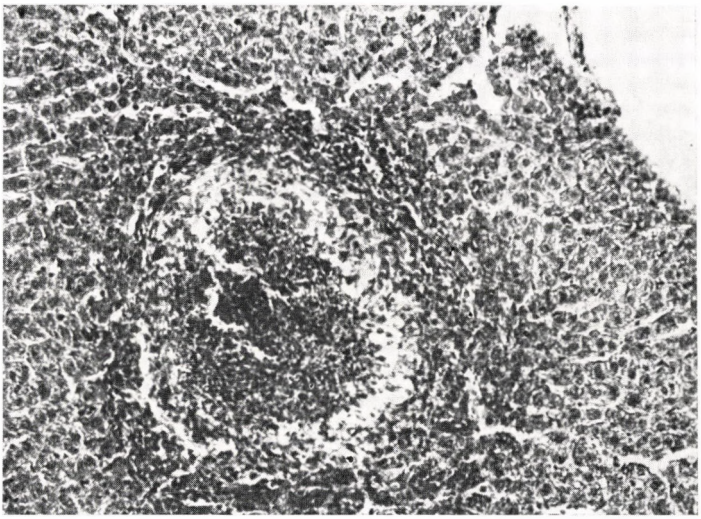

*Fig. 3.* Patch of granular eosinophilic and basophilic debris in liver of rabbit 4 days after infection. Haem.–eos. ×200

ation of fibroblasts. The lumen of some bile ducts contained eosinophilic granules. The macroscopic picture showed numerous grayish white foci 7 weeks after the infection.

Rabbit: Liver examined 1 week after the infection exhibited small interstitial patches of lymphocytes and fibroblasts. Most of the bile ducts were dilated, exhibited thickened fibrosed wall and contained granular eosinophilic debris inside their lumen (Fig. 6). Liver examined 3 weeks after the infection was congested and presented large areas of coagulative necrosis with thrombosed portal veins. Hepatic sinusoids contained fairly numerous siderocytes. Few fibrin threads hanged from the hepatic capsule. Excessive amount of hemosiderin occupied the sinusoids, cytoplasm of macrophage and hepatic cells 7 weeks after the infection. Fatty change of the hepatic cells and granulomatous nodules were present together with focal granular eosinophilic debris (Fig. 3). The macroscopic picture presented white nodules of 3—7 mm in diameter 7 weeks after the infection.

Chicken: Larvae were present among hepatic cells 1 and 3 weeks after the infection. They were surrounded in some areas with fibroblasts. Interstitial aggregations of lymphocytes alone, or together with eosinophils and foreign body type giant cells were present. These aggregations caused pressure atrophy of the surrounding hepatic cells (Fig. 4). Patches of necrotic hepatic cells, lymphocytes, heterophils and giant cells were frequently seen near the

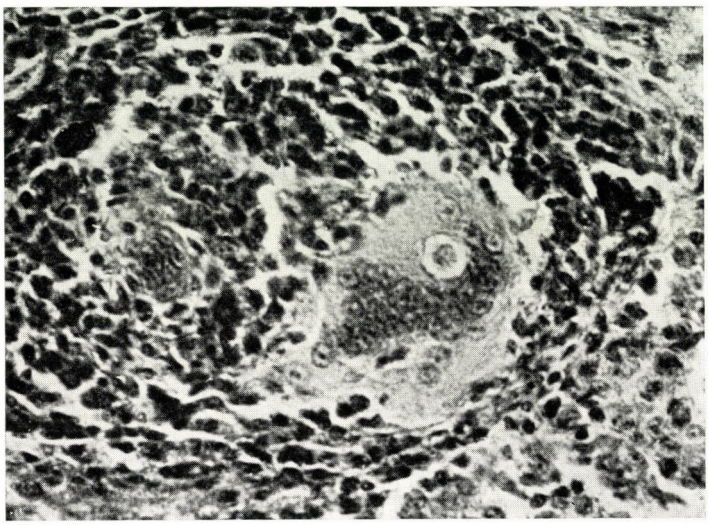

*Fig. 4.* Granulomatous nodule causing pressure atrophy of hepatic cells in a chicken 7 days after infection. Haem.–eos. ×1000

portal tract 7 weeks after the infection. The epithelial lining of the bile ducts was desquamated. The wall of some bile ducts was thickened with fibrous tissue.

*Lungs:*

Rat: Larvae were present in pulmonary tissue 1 week after the infection. These larvae were surrounded with eosinophils, macrophages and giant cells which were frequently encapsulated with fibrous tissue infiltrated with eosinophils (Fig. 5).

Guinea-pig: Larvae were present 2 and 3 weeks after the infection. The inflammatory reaction around the larvae consisted of fibrous tissue surrounded with numerous eosinophils, few neutrophils and some macrophages and giant cells, both of the foreign body and the Langhan's types (Fig. 6). Diffuse interstitial fibrous tissue proliferation and eosinophilic infiltration

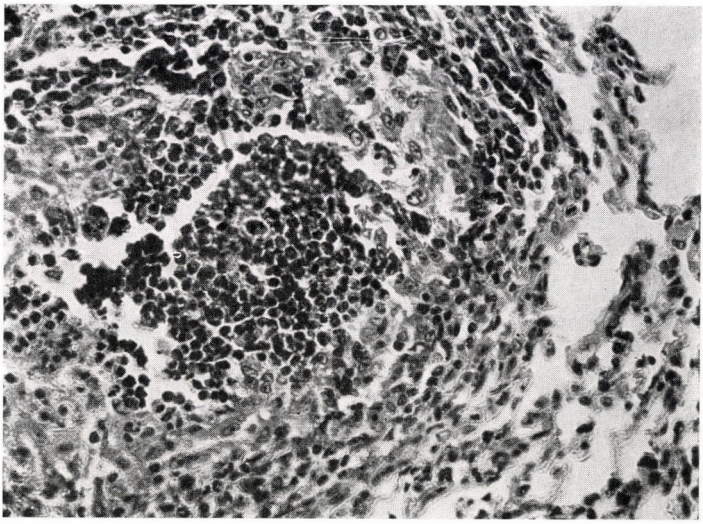

*Fig. 5.* Patches of eosinophils, macrophages and giant cells of foreign body type in lungs of rat 7 days after infection. Haem.–eos. ×400

with collapsed and emphysematous pulmonary tissue were seen. Nodules with necrotic eosinpohilic center surrounded by macrophages, giant cells and eosinophils were frequently observed. Peribronchial smooth muscles were hypertrophic, and the wall of some arterioles was thickened, hyalinized,

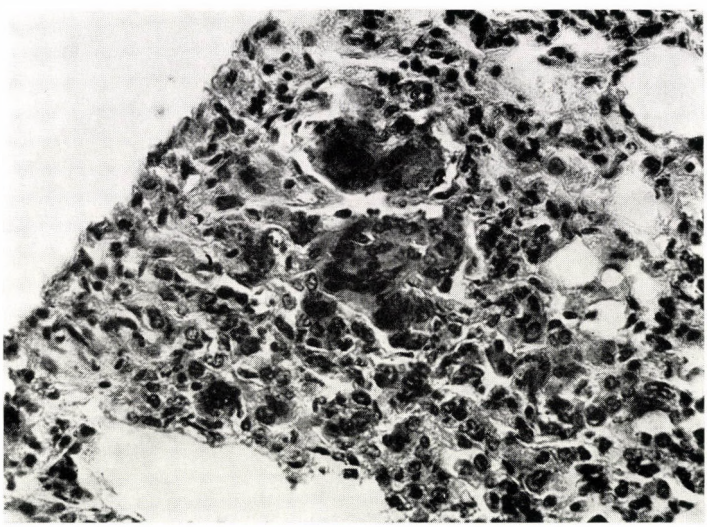

*Fig. 6.* Eosinophils, macrophages and giant cells both of the foreign body and Langhans types, lymphocytes the lungs of guinea pig 7 days after infection. Haem.– eos. ×400

vacuolar and infiltrated with few eosinophils. The visceral pleura showed focal thickenings due to fibrous tissue proliferation. The epithelial lining of most bronchioles was hyperplastic and partially desquamated, and the lumen contained mucous exudate. Macroscopically, the lungs presented lobular pneumonia 1 week after the infection. Most of the lobules were either red or brown.

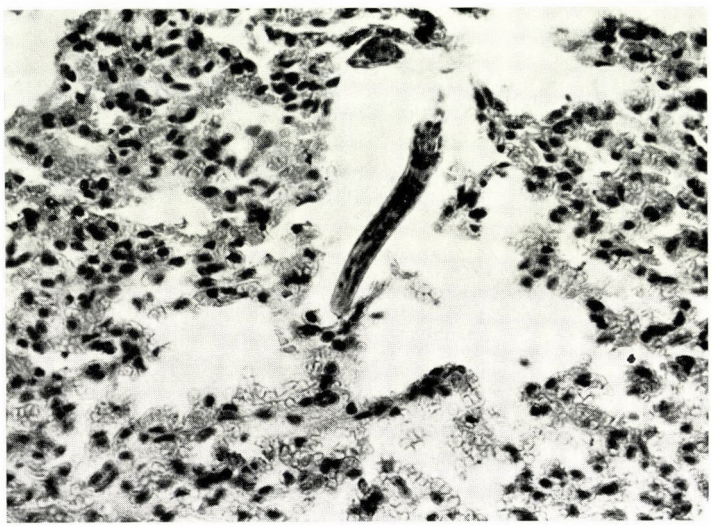

*Fig. 7.* Larva and interstitial fibrous tissue proliferation in lungs of rabbits 21 days after infection. Haem.–eos. ×400

Rabbit: Larvae were present near the pleura (Fig. 7) 3 weeks after the infection. Small haemorrhages and fibrous tissue proliferation were present particularly under the pleura. The pulmonary blood vessels were congested. A perforation in the pleura was represented with thickened borders due to fibrous tissue proliferation.

The macroscopic picture showed fairly numerous petechial haemorrhages throughout the pulmonary tissue.

Chicken: The pulmonary tissue showed necrotic patches surrounded by macrophages, fibroblasts and giant cells. Lymphocytic infiltrations appeared as nodules in the interstitial tissue or as aggregations in the lamina of the bronchioles (Fig. 8) with desquamated epithelial lining. Peribronchial and perivascular infiltration with eosinophils were prominent.

*Kidneys:*

Guinea-pig: Larvae were present among cortical renal tubules 3 weeks after the infection (Fig. 9). Patches of lymphocytes, plasma cells, eosinophils

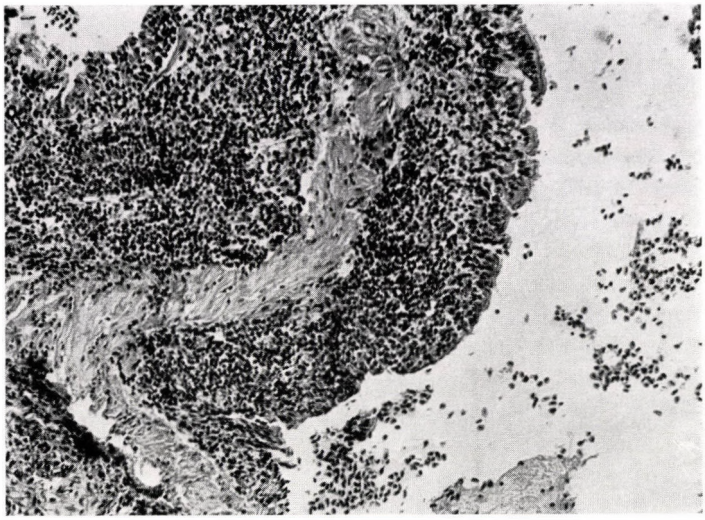

*Fig. 8.* Lymphocytic infiltration in the lamina propria of bronchioles with desquamated epithelial lining in chicken 49 days after infection. Haem.–eos. ×200

and fibroblasts were scattered in the cortex (Fig. 10) or under the epithelial lining of the renal pelvis (Fig. 11). Some of these patches contained larvae. Bowman's capsules of glomeruli adjacent to the inflammatory areas were thickened. Hyaline casts were present inside the renal tubules 10 weeks after the infection. The macroscopic picture showed few whitish foci of 1—2 mm diameter in the cortex 7 weeks after the infection.

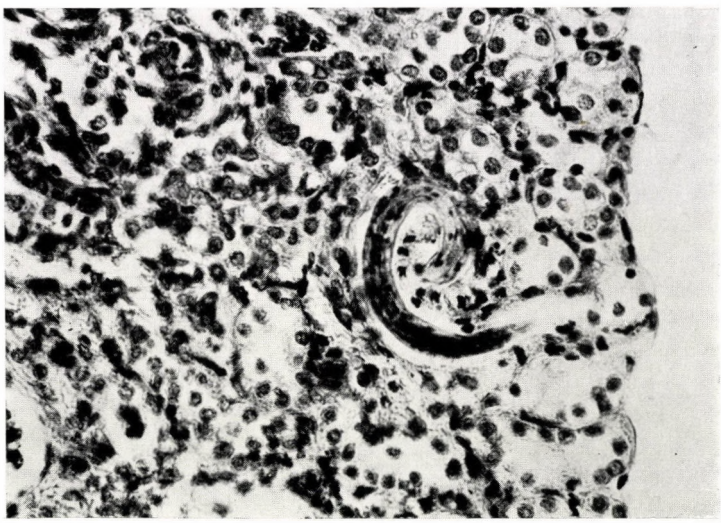

*Fig. 9.* Larva among renal tubules in guinea pig 21 days after infection. Haem.– eos. ×400

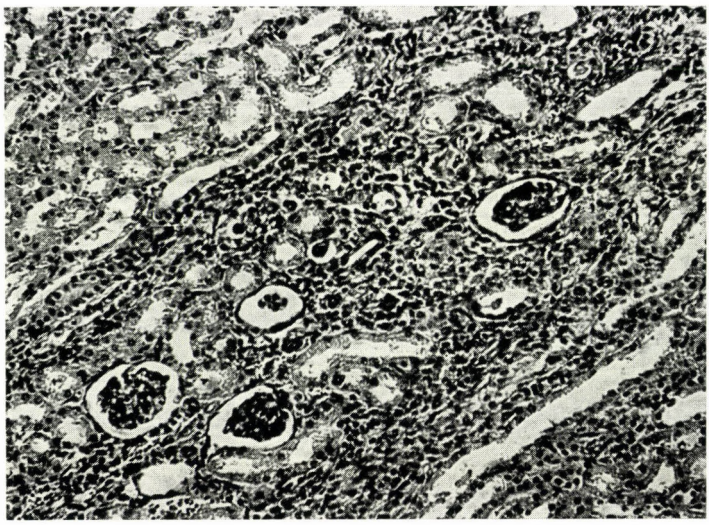

*Fig. 10.* Aggregation of fibroblasts, eosinophils, lymphocytes and plasma cells in renal cortex of guinea pig 49 days after infection. Haem.–eos ×200

Rabbit: Few white foci of about 2 mm in diameter were present in the renal cortex 7 weeks after the infection.

Chicken: Patches of lymphocytes, eosinophils and fibroblasts were seen mostly near the renal capsule.

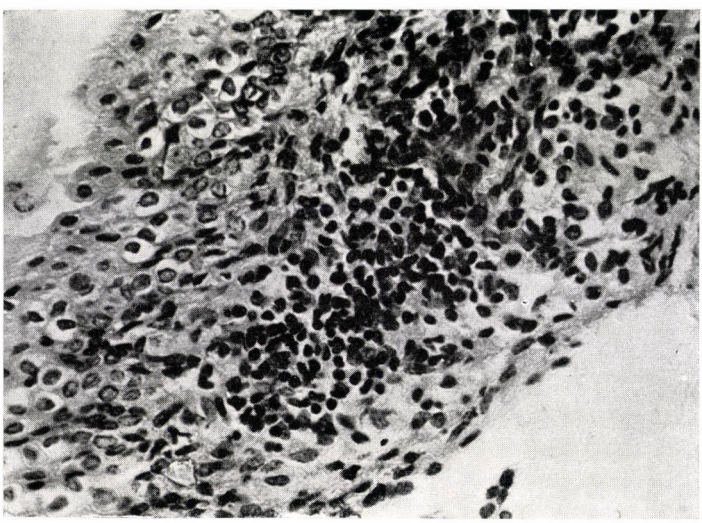

*Fig. 11.* Lymphocytic infiltration under the epithelial lining of renal pelvis in guinea pig 14 days after infection. Haem.–eos. ×400

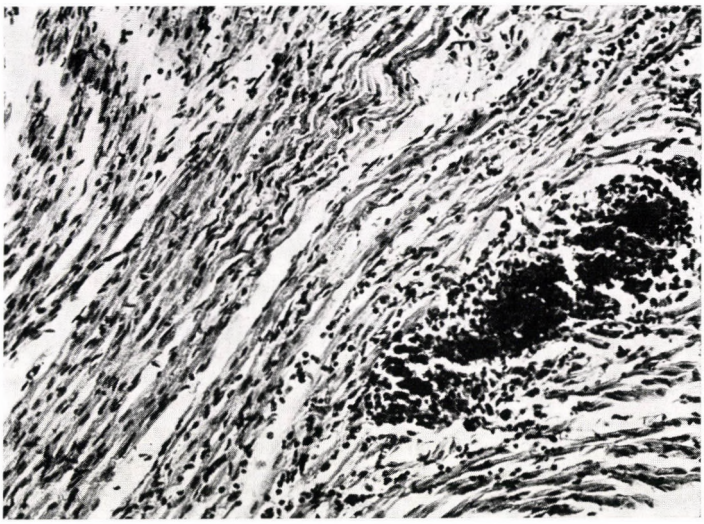

*Fig. 12.* Eosinophilic granular necrotic patches in myocardium of guinea pig 49 days after infection. Haem.-eos. ×200

*Myocardium:*

Rat: Patches of lymphocytic infiltration and hyalinized muscles were seen 7 and 10 weeks after the infection. Epicardium displayed aggregations of lymphocytes.

Guinea-pig: Larvae were present among the muscle fibres 3 weeks after

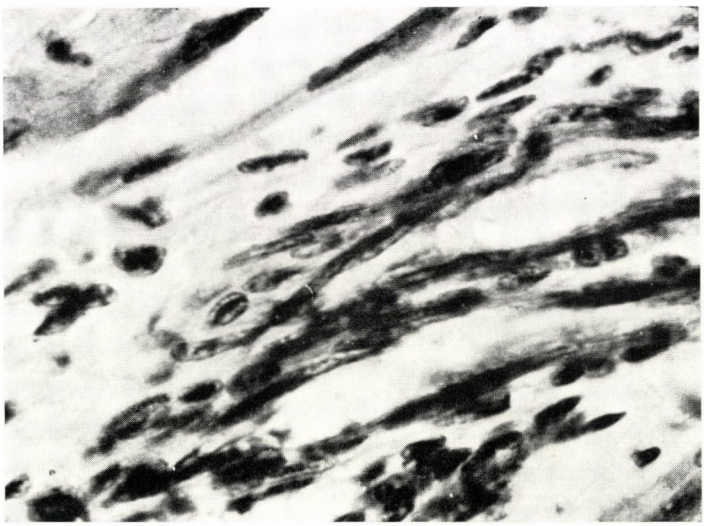

*Fig. 13.* Showing Anitschkow myocytes in wall of coronary arterioles in guinea pig 14 days after infection. Haem.-eos. ×1000

the infection. These larvae were degenerated and surrounded with young fibroblasts and eosinophils. Patches of haemorrhage, necrosis and eosinophils, fibroblasts and giant cells were present among muscle fibres (Fig. 12) particularly in the papillary muscles of the left ventricle. Histiocytes resembling to Anitschkow myocytes (Fig. 13) were prominent in the wall of coronary arterioles. The latter were frequently surrounded with edema, eosinophils and fibroblasts. Fibrinous threads projected from the epicardium 7 weeks after the infection.

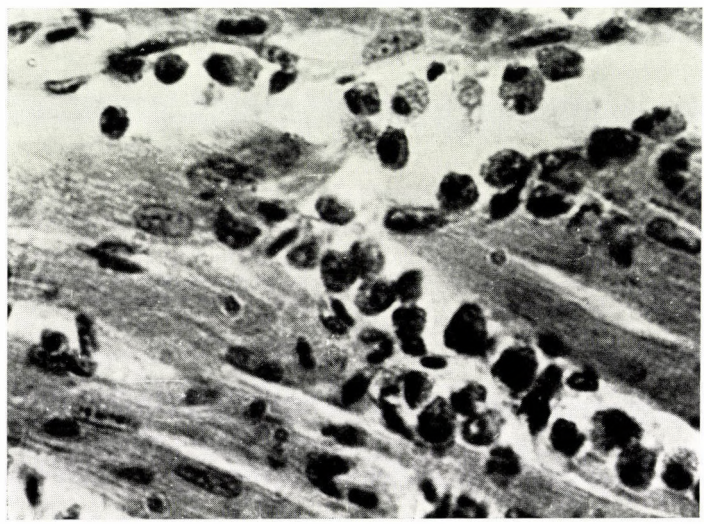

*Fig. 14.* Showing infiltration of heterophils and lymphocytes among muscle fibres of myocardium of chicken 49 days after infection. Haem.-eos. ×1000

Chicken: The myocardium was infiltrated with heterophils and lymphocytes 1 and 7 weeks after the infection (Fig. 14). Epicardium was edematous and infiltrated by the above mentioned cells. Nuclei of the cardiac muscle cells were generally similar to the Anitschkow myocytes particularly in the wall of the left ventricle.

*Brain:* No larvae were found.

Rat: Meninges were congested and haemorrhagic 2 weeks after the infection. White matter of the cerebellum presented few haemorrhagic patches, and the Purkinje cell layer was edematous.

Guinea-pig: Areas of leukomalacia in the cerebrum and cerebellum were seen 1, 2 and 3 weeks after the infection. The Purkinje cell layer was edematous. Pia mater was focally infiltrated with lymphocytes (Fig. 15).

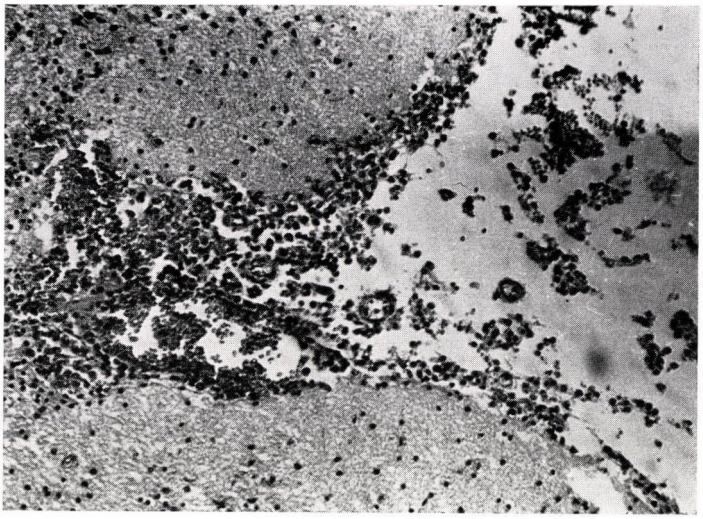

*Fig. 15.* Focal infiltration with lymphocytes in pia mater of guinea pig 21 days after infection, Haem.–eos. ×200

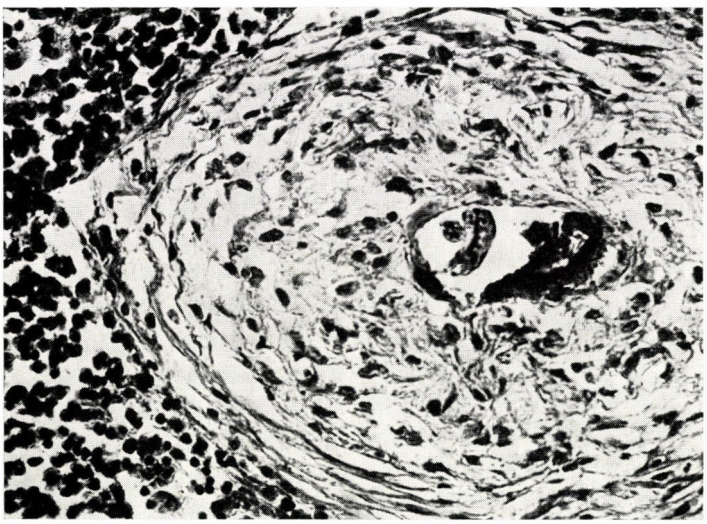

*Fig. 16.* Showing larvae surrounded with a thick fibrous tissue capsule in submandibular lymph node of rat 49 days after infection. Haem.–eos. ×1000

Rabbit: Areas of malacia in the white matter were present 3 weeks after the infection.

*Spleen:* No larvae were found.

Rat: Megakariocytes were numerous 1 and 2 weeks after the infection. The Malpighian corpuscles were hyperplastic and patches of eosinophils were present. Few megakariocytes were seen 7 and 10 weeks after the infection.

Guinea-pig: Aggregations of eosinophils were present 1 and 7 weeks after the infection.

Rabbit: Most Malpighian corpuscles were depleted of lymphocytes 1 week after the infection.

Chicken: Few heterophils accumulated in the red pulp, and the Malpighian corpuscles were hyperplastic 2 weeks after the infection.

*Lymph node:*

Rat: Larvae were present in the submandibular lymph node 7 weeks after the infection. A thick wall of fibrous tissue surrounded the larvae (Fig.16).

## Discussion

SPRENT (1954) demonstrated that migration of ascarid larvae may follow the tracheal route, the somatic one or both. The present findings support those of WARREN (1971) who demonstrated a somatic migration for *Neoascaris vitulorum* in mice. Similar results were reported also by SCHWARTZ (1922) who found larvae of *Neoascaris vitulorum* in the liver, lungs and kidneys of guinea pigs which were fed embryonated eggs.

The lesions caused by the migrating larvae were most extensive in guinea pig, moderate in rat and mild in chicken and rabbit. These variations of lesions may be due to the number of larvae invading the tissue in the various hosts, as it is known that brain of mouse is more susceptible to invasion with larvae of *Toxocara canis* than that of rat or guinea pig (SPRENT, 1955). Such lesions may also be attributed to allergic reactions (ERSHOV and NAUMICHEVA, 1966), or pathogenic microorganisms carried by the larvae.

The presence of larvae was almost consistent in the liver, lungs and kidneys, rare in myocardium and lymph nodes, and was not seen in brain and spleen.

Lesions in various organs were manifested by granular eosinophilic and basophilic necrotic debris and inflammatory cells, mainly eosinophils, lymphocytes, fibroblasts, macrophages and giant cells. This is in accordance with

SPRENT (1958) who stated that visceral larva migrans cause multiple granuloma formation in various organs particularly in the liver and lungs.

Migration of the larvae in various organs induced lesions of variable significance. Hepatic lesions were characterized by being mild in rat, showing degenerative and necrotic changes in guinea pig and rabbits; and by aggregations of lymphocytes in chickens. The numerous eosinophils inside the congested portal vein suggest eosinophilia which was also observed by SMITH and BEAVER (1953) in a child infected with eggs of *Toxocara canis* and MERCER et al. (1950) who reported a syndrome of chronic eosinophilia and eosinophilic infiltration of the liver, containing ascarid larvae in a child.

The pulmonary lesion manifested by granulomatous pneumonia was in line with McCRAW and LAUTENSLAGER's observations (1971) who reported an outbreak of foetal pneumonia in calves naturally infected with large number of *Ascaris suum* larvae (DEWAELE et al., 1972). The absence of haemorrhagic areas from pulmonary tissue may indicate that larvae did not break through air-sacs as the diameter of larvae is less than 0.02 mm (FÜLLEBORN, 1925). This finding may support the somatic migration demonstrated by WARREN (1971) in mice.

The presence of larvae among renal tubules is also indicative of somatic migration, such larvae may cause albuminuria as suggested by the presence of hyaline casts inside the lumina of renal tubules.

The cardiac lesions were outstanding, but did not reach the degree of interference with the cardiac function, as no general venous congestion was noticed. However, the fibrin threads and lymphocytes in the epicardium indicate the presence of pericarditis caused by larvae which pierced the epicardium. BECROFT (1964) reported fatal myocarditis in mice due to *Toxocara canis*.

The absence of ascaris larvae in the brain is usually attributed to tracheal migration (SPRENT, 1952), but the present work as well as that of WARREN (1971) suggest somatic migration. Yet, the presence of haemorrhagic patches in meninges and brain suggests that larvae could have migrated through the brain.

The finding of larvae inside the submandibular lymph node shows that the larvae could have migrated through lymphatics. This is in accordance with MATOFF (1949) who found ascarid larvae in mesenteric lymph nodes, and concluded larval migration through the thoracic duct. Lymph nodes may seem to react with excessive fibrous tissue production as suggested by the thick fibrous tissue capsule around the larva.

Egyptian farmers are usually in contact with the feces of cattle in the form of fertilizer or fuel. This may contaminate their food and expose them to infection with *Neoascaris vitulorum*. Another source of infection may be the consumption of unboiled milk from infected animals. WARREN (1971) concluded

that calves acquire infection from their dams through milk. Consumption of such unboiled milk by man may expose him to repeated infection with larvae of *Neoascaris vitulorum*. Whether such repeated infection may occur and cause cirrhosis and ascites, needs further investigation.

*Acknowledgements.* The authors express their deep gratitude to Prof. Dr. M. SHAMS EL-DIN Moustafa, head of Pathology, Microbiology and Parasitology Department and Dean of the Faculty, for his great interest through this investigation.

Thanks are also due to M. F. A. FAHMY, assistant Professor of Histology, for his kind help in preparing the microphotographs.

## Summary

Laboratory animals and chickens were experimentally infected with embryonated eggs of *Neoascaris vitulorum*.

Larvae of this parasite were present in the liver, lungs, heart, kidneys and lymph nodes. Lesions were mainly granulomatous and more severe in guinea pigs. The authors suggest that larvae of *Neoascaris vitulorum* may cause visceral larva migrans in human.

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# RESEARCH IN COCCIDIA AND OTHER *APICOMPLEXA*
## A PROGRESS REPORT

By

B. MÜLLER and E. SCHOLTYSECK*

Dept. of Protozoology, Institute of Zoology, University Bonn

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### Introduction

Electron microscopy of *Coccidia* is a rather young branch of protozoological research. The first investigations were carried out less than 20 years ago. With the improvement of fixation and embedding methods for electron miscroscopy, the quality of these investigations and results increased considerably. Ultrastructural approaches were of special importance for this group of *Protozoa*, because their small size makes them a poor subject for light microscopy. After those new techniques were applied, numerous and often surprising results were obtained in a short time. Therefore, the taxonomy of the subphylum *Apicomplexa* (= Sporozoa + Piroplasmids) has been revised and is no longer based on developmental characteristics, but on ultrastructural criteria.

Our research team at Bonn has been involved in ultrastructural investigations on *Apicomplexa (Gregarinia, Coccidia, Haemosporidia, Piroplasmida)* for more than 15 years. A review over the last 12 years will be given herein.

This report is based on and is made possible through the collaboration and cooperation of the following investigators and departments: B. CHOBOTAR, A. J. DEVOS, J. V. ERNST, D. M. HAMMOND (Department of Zoology, Utah State University, Logan, Utah, USA), M. L. MINER (Department of Veterinary Science, Utah State University, Logan, Utah, USA), R. G. STROUT (Department of Animal Sciences, University of New Hampshire, Durham, New Hampshire, USA), J. SENAUD (Laboratoire C.N.R.S. "Biologie des protistes", Université de Clermont-Ferrand, Aubière, France), L. PELLÉRDY (Research Institute of Veterinary Science, Hungarian Academy of Sciences, Budapest, Hungary), O. KEPKA (Zoologisches Institut, Universität Graz, Austria.), G. PIEKARSKI (Institut für Medizinische Parasitologie, Universität Bonn, BRD), K. FRIEDHOFF, G. WEBER (Institut für Parasitologie, Tierärztliche Hochschule Hannover, BRD), R. GÖNNERT, A. HABERKORN (Institut für Parasitologie, Farbenfabriken Bayer AG., Wuppertal, BRD), D. SPIEKER (Forschungsstelle für Jagdkunde und Wildschadenverhütung, Forsthaus Hardt, Bonn-Niederholtorf, BRD), G. HELLER, H. MEHLHORN, A. ROMMEL, D. SCHÄFER, E. SCHULTE, W.-H. VOIGT, B. VOLKMANN (Abteilung für Protozoologie,

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## Fine structure of deveplomental stages

Originally, a major part of our investigations was concerned with the ultrastructure of different coccidian developmental stages. The results of these observations were subsequently used as a basis for further investigations in transformation of different stages, in developmental processes, in cytochemistry and in other problems. Furthermore, ultrastructural research on various species allowed and led to generalizations and conclusions important not only for all coccidia and their taxonomic subgroups, but also for related sporozoan groups.

An example of these comparative investigations is the research on merozoites of *Eimeria perforans* and *E. stiedai* and on intracellular trophozoites (merozoites) of *Toxoplasma gondii* (SCHOLTYSECK and PIEKARSKI, 1965). Ultrastructural features common in merozoites of both *Eimeria* species are the pellicle consisting of an outer unit membrane with an underlying complex of 2 inner unit membranes, anterior and posterior circular openings in the inner membrane complex, and a conoid located in the anterior opening. Further characteristic organelles are subpellicular microtubules, paired organelles (rhoptries) and micronemes in connection with the conoid. A Golgi-apparatus is usually located at the anterior periphery of the nucleus.

A comparison with intracellular stages of *T. gondii* showed the same structure and arrangement of organelles as in *Eimeria* merozoites, with only quantitative differences (SCHOLTYSECK and PIEKARSKI, 1965). We then suggested the inclusion of *T. gondii* into the coccidia, based on the high similarity of these results.

Other studies dealt with the ultrastructure of merozoites of other species and genera. While investigations on *E. falciformis* (PELLÉRDY et al., 1971) and *E. tenella* (SCHOLTYSECK and MEHLHORN, 1970; STROUT and SCHOLTYSECK, 1970) confirmed earlier findings on the general ultrastructure of eimerian merozoites, merozoites of *Aggregata eberthi (Eimeriorina, Aggregatidae)* (HELLER, 1970) and *Babesia* spp. *(Apicomplexa*, class *Piroplasmida)* showed distinct differences (FRIEDHOFF and SCHOLTYSECK, 1968, 1969, 1972).

Merozoites of eimerian species have 22, 24 or 26 subpellicular microtubules (depending on the species) (SCHOLTYSECK and MEHLHORN, 1970), whereas about 60 microtubules are found in *A. eberthi* (HELLER, 1969) and 32 are found in *Babesia bigemina* (FRIEDHOFF and SCHOLTYSECK, 1969). At the apical pole, the inner membranes leave an opening for the conoid in *Eimeria* and *Aggregata* spp.; a conoid is not present in *Babesia* spp. (SCHOLTYSECK

et al., 1970) and in *Haemosporina*. In species of the genus *Eimeria*, often 2 rhoptries (paired organelles) and many micronemes are located in close proximity to the conoid, whereas *A. eberthi* shows more than 2 rhoptries. In *Babesia* species, 2—3 rhoptries and many micronemes are located at the site of the anterior opening of the inner membrane complex (SCHOLTYSECK and MEHLHORN, 1970).

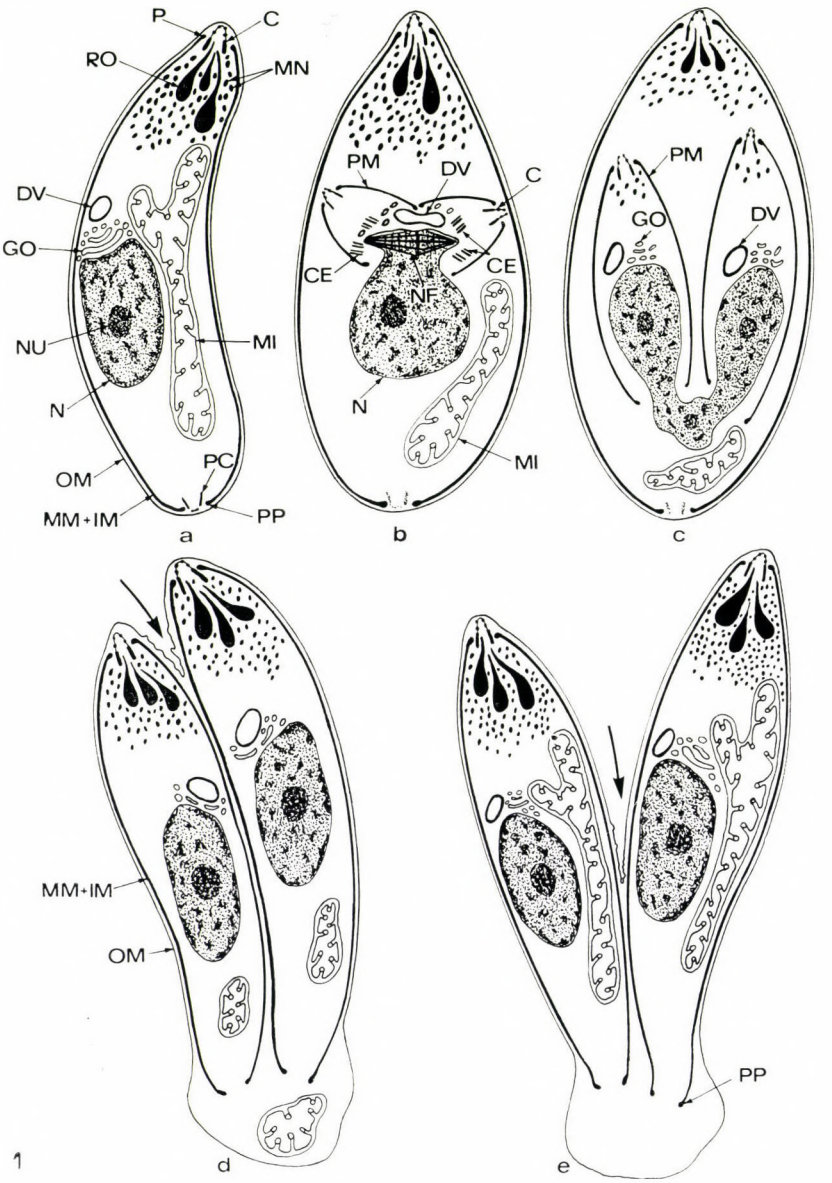
Recently, comparative investigations on cyst-stage merozoites of *Sarcocystis tenella*, *Besnoitia jellisoni*, *Frenkelia* sp. and *T. gondii* (MEHLHORN and SCHOLTYSECK, 1973; MÜLLER et al., 1973c; SCHOLTYSECK et al., 1970) confirmed the establishment of a close relationship between these genera and their inclusion into the suborder *Eimeriorina*. Like merozoites of other *Eimeria* species, they have the typical 3-layered pellicle with a conoid in the anterior pole and 22 subpellicular microtubules. More than 2 rhoptries are usually present. Endodyogeny as the exclusive way of multiplication is common in all 4 genera (KEPKA and SCHOLTYSECK, 1970a, b). Except in *Frenkelia* sp., the cyst has been shown either to have developed from a parasitized host cell or to be a parasitized host cell (in *Besnoitia jellisoni*) (MÜLLER et al., 1973d).

Yet another interesting aspect of the investigations on these 4 genera is the functional morphology of the infective stages. In the developmental sequence of metrocyte — intermediate cell — merozoite inside the cyst, the conoid becomes longer and its diameter decreases, becoming more and more a tapered ("pin-pointed") penetration organelle. In addition to this shape change of the conoid, which can be seen in *S. tenella* and *Frenkelia* sp., the number of rhoptries and micronemes also increases in the above mentioned developmental sequence (MÜLLER et al., 1973c). Species-specific ultrastructural characteristics were found for all 4 parasites.

Not yet understood is the role of the microtubules in the motility of merozoites. Although the loss of motility in schizonts and gamonts coincides with the disappearance of microtubules on electron micrographs of these stages, only hints for the possible function of microtubules can be given. One of these hints is the dislocation of subpellicular microtubules observed in merozoites of *E. falciformis*, where they lose the connection to the pellicle and extend across the inner cytoplasm in bending (flexing) merozoites (SCHOLTYSECK et al., 1972a, b).

Of similar interest were comparative studies on macrogamonts of different eimerian species (MEHLHORN, 1972a, b; SCHOLTYSECK, 1962; SCHOLTYSECK et al., 1969; SCHOLTYSECK and HAMMOND, 1970; SCHOLTYSECK et al., 1966; SCHOLTYSECK et al., 1971a; SCHOLTYSECK et al., 1971b; SCHOLTYSECK and SPIEKER, 1964), of *Klossia helicina (Adeleorina)* (SCHULTE, 1971b; VOLKMANN, 1967) and of *A. eberthi* (HELLER, 1969). After the transformation of merozoites into usually immotile macrogamonts, subpellicular microtubules are lost and the amount of reserve polysaccharide decreases, at least in *E.*

*perforans* (SCHOLTYSECK, 1964; SCHOLTYSECK et al., 1971a). In macrogamonts of all species, the typical sporozoan pellicle consists of an outer unit membrane and a complex of 2 inner unit membranes. In the process of maturation, i.e. during the development into macrogametes, several ultrastructural changes occur. In most eimerian species, the 3 unit membranes of the pellicle are reduced to either 2 outer membranes with underlying osmiophilic material



— in *E. bovis* (SCHOLTYSECK and HAMMOND, 1970; SCHOLTYSECK et al., 1966) and *E. maxima* (MEHLHORN, 1972; SCHOLTYSECK et al., 1971a) —, or to one outer unit membrane with underlying osmiophilic plaques — in *E. tenella* (SCHOLTYSECK et al. 1969a), *E. stiedai* (SCHOLTYSECK et al., 1966), *E. perforans* (SCHOLTYSECK, 1962; SCHOLTYSECK et al., 1966), and *E. falciformis* (SCHOLTYSECK et al., 1971a; SCHOLTYSECK et al., 1971b) —, or to a middle membrane with over- and underlying osmiophilic material — in *E. auburnensis* (SCHOLTYSECK et al., 1966). In all cases, the osmiophilic material is interpreted as a relict of the other unit membranes. The change in pellicle ultrastructure seems to occur at different growth stages in different species. In *A. eberthi* (HELLER, 1969) and in *K. helicina* (SCHULTE, 1971b; VOLKMANN, 1967), this degeneration of the pellicle does not occur.

All eimerian species show 2 different kinds of wall-forming bodies (called WFB), which develop subsequently during the maturation process (SCHOLTYSECK et al., 1971b; SCHOLTYSECK and STROUT, 1968). The type built first lies in a vacuole which is derived from the Golgi-apparatus and is of a spongeous structure. As these wall-forming bodies give rise to the inner part of the oocyst wall, they are called WFB II. Occasionally, 2 or more WFB II are located inside one vacuole. During the course of maturation, homogeneous electron-dense, membrane-bound vesicles, called WFB I, develop out of smaller vesicles and assume a peripheral position between the pellicle and the WFB II. After fertilization, the WFB I flow together and form the outer layer of the oocyst wall (SCHOLTYSECK et al., 1969a, b).

Only one type of wall-forming bodies is present in *A. eberthi* (HELLER, 1969). As no special oocyst wall is formed in this species, the wall-forming bodies, which resemble the WFB I-type of *Eimeria* spp., give rise to the spore wall. In *K. helicina* (SCHULTE, 1971b; VOLKMANN, 1967), organelles resembling the WFB II of the eimerian type have been found, but their participation in the formation of oocyst or spore walls has not been shown.

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*Fig. 1.* A diagrammatic representation of endodyogeny. a) Merozoite prior to division. b) Formation of an intranuclear spindle apparatus at the anterior pole of the nucleus. The Golgi-apparatus and the Golgi-adjunct are dividing, the anlagen of the two developing daughter cells, with conoids and centrioles, become visible. The inner membrane complex is formed *de novo*. c) Development of daughter cells continues. U-shape of the nucleus, with one protrusion into each of the two daughter cell anlagen. d) Nuclear division completed. At the anterior pole, the outer pellicular membrane begins to invaginate, leading to the separation of the daughter cells. e) Progressing separation. The outer pellicular membrane and, especially in the posterior part, the inner membrane complex of the mother cell have been taken over by the two developing daughter cells.

Abbrevations: C, conoid; CE, centriole; DV, dark-walled vesicle (Golgi-adjunct); GO, Golgi-apparatus; IM, inner pellicular membrane; MI, mitochondrion; MM, middle pellicular membrane; MN, micronemes; N, nucleus; NF, intranuclear spindle fibrils; NU, nucleolus; OM, outer pellicular membrane; P, polar ring; PC, posterior cone; PM, pellicle (inner membrane complex) of the developing daughter cell anlagen; PP, posterior polar ring; RO, rhoptries

At the surface of macrogamonts of all species, special peripheral structures extend into the parasitophorous vacuole surrounding the parasite (SCHOLTYSECK et al., 1971b). Their form and function will be considered in the chapter on host–parasite relationship.

## Developmental processes

This chapter will be divided in two parts. The first, dealing with asexual development, will consider schizogony (merogony) and sporogony; the second part — sexual development — will deal with gamogony.

### *Ia. Schizogony (merogony)*

After having invaded a host cell, sporozoites of all *Eucoccidiorida* (*Schizococcidia* Grell) undergo a growth phase which terminates in the development of two to several thousand merozoites from a single schizont or from schizont subunits. These merozoites usually invade new host cells, where schizogony is repeated. After a species-specific number of merogonies (schizogonies), merozoites develop into sexual forms. This process will be considered later.

In both *E. perforans* and *E. stiedai*, schizogony follows a similar pattern (HELLER and SCHOLTYSECK, 1971; SCHOLTYSECK, 1965a, b). Nuclear divisions occur in the growing schizont, until about 50 nuclei are evenly distributed throughout the cytoplasm. Then, each single nucleus with the surrounding cytoplasm is separated from the plasmodium by flat, membrane-bound cisternae. It has not yet been determined whether these cisternae originate from the endoplasmic reticulum (probably with some participation of the Golgi-apparatus) or whether they represent cross-sections of deep pellicular invaginations. At the moment, a combination of both processes seems to be most likely. Finally, each nucleus plus the surrounding cytoplasm develop into a merozoite, with the inner membrane complex being formed *de novo* and the outer pellicular membrane being derived from the outer membrane and/or endoplasmic membranes of the schizont.

Additional information on the early developmental processes is available from investigations on *E. falciformis* (PELLÉRDY et al., 1971), *E. contorta* (MÜLLER et al., 1973b), *E. canadensis* (MÜLLER et al., 1973a), and *E. tenella* (STROUT and SCHOLTYSECK, 1970). In all these species, the sporozoite first loses its shape and rounds up into a uninucleate schizont (trophozoite). Second, the inner membrane complex breaks down, while nuclear divisions take place. In *E. canadensis* (MÜLLER et al., 1973a), nuclear divisions sometimes occur before the sporozoite rounds up. This leads to the formation of a sporozoite-shaped schizont. In the so-called small schizonts of *E. stiedai* (HELLER, 1971),

nuclear divisions and the development of new merozoites occur in merozoites of the previous generation, which are then called merozoite-shaped schizonts.

*E. canadensis* differs from the other species mentioned in regard to the formation of merozoites (MÜLLER et al., 1973a). Here, several thousand merozoites are formed only at the surface — not throughout the cytoplasm — of several schizont subunits. This means that the formation of merozoites is more like a growing process at the surface than like an invagination of the outer membrane around the interiorly developing merozoite as in other species.

In our investigations on *Frenkelia* sp., we found that this species in its cyst stage multiplies by means of endodyogeny (KEPKA and SCHOLTYSECK, 1970a; KEPKA and SCHOLTYSECK, 1970b). This process, known from *Toxoplasma*, *Besnoitia*, and *Sarcocystis*, starts out from a uninucleate merozoite (or metrocyte, or intermediate cell) (MÜLLER et al., 1973a, b, c). Shortly before nuclear division sets in, 2 pairs of centrioles can be seen at one pole of the nucleus. Then, an intranuclear spindle apparatus is formed, the Golgi-apparatus and the Golgi adjunct (dark-walled vesicle) divide, and the inner membrane complex and the conoids of 2 daughter cells are formed in close connection to the centrioles and the dividing nucleus (KEPKA and SCHOLTYSECK, 1970b; Fig. 1). The daughter cells grow and develop inside the mother cell, until they finally take over the outer pellicular membrane of the metrocyte and become free. An event, similar to endodyogeny, was found in *E. stiedai* (HELLER, 1971) and, recently, in *E. tenella*. Here, the last nuclear division during merogony can be interpreted as an endodyogeny. The conclusion of these studies is that endodyogeny may be considered as primary agamic reproduction (SCHOLTYSECK, 1973). Additional detailed information on the role of the centrioles and the nuclear spindle apparatus was obtained for *E. falciformis* and *E. maxima* (MEHLHORN et al., 1973).

### *Ib. Sporogony*

Investigations on the formation of sporozoites are hindered to a rather large extent by the barriers to fixation media built up by the oocyst and sporocyst membranes. Occasional findings in *Aggregata eberthi* (HELLER, 1969) and *Klossia helicina* (VOLKMANN, 1967) gave some information on the ultrastructure of spores, but are not considered sufficient for any statement on the developmental processes involved.

## *II. Gamogony*

As the maturation process of macrogametes was already mentioned in the chapter on ultrastructure of developmental stages, this section will deal only with the development of microgametes, i.e. with microgametogenesis.

Our investigations have been carried out on *E. auburnensis* (HAMMOND et al., 1969; HAMMOND et al., 1969), *E. bovis* (HAMMOND et al., 1967a), *E. falciformis* (SCHOLTYSECK et al., 1973), *E. maxima* (MEHLHORN, 1972a), *E. perforans* (HAMMOND et al., 1967b; SCHOLTYSECK, 1965), *E. stiedai* (HAMMOND et al., 1967a, b), and on *A. eberthi* (HELLER, 1970a). In all cases, microgamonts undergo numerous nuclear divisions which closely resemble those described for merogony (schizogony). However, the resulting nuclei are not distributed evenly as in schizonts, but form a line adjacent to the microgamont's surface (SCHOLTYSECK et al., 1972). This surface, which previously underwent a reduction of the inner membrane complex, shows many micropores, with the exception of *E. stiedai* (HAMMOND et al., 1967a). In *E. auburnensis* (HAMMOND et al., 1969; HAMMOND et al., 1967b), the surface is increased considerably by deep pellicular infoldings and fissures. A similar process in *E. maxima* (MEHLHORN 1972; SCHOLTYSECK, 1963a) and *A. eberthi* (HELLER, 1970a) results in the formation of several blastophores (gamont-subunits), thus increasing the surface area and the number of microgametes formed to several thousands. In most species investigated, this first (growth) phase is completed before the second (differentiation) phase starts simultaneously for all gametes. Only in *E. falciformis* was a successive development of microgametes observed (SCHOLTYSECK et al., 1973).

The differentiation process begins with the appearance of 2 centrioles and a mitochondrion adjacent to each nucleus. Then, the surface immediately above each nucleus protrudes and gradually elevates, incorporating the centrioles plus developing flagella, the nucleus and the mitochondrion. During this process, the nucleus undergoes an interesting differentiation process. Most of the karyoplasm condenses in the area next to the centrioles, while the nucleus becomes slender and cylindrical. While in *E. perforans* (SCHOLTYSECK, 1965a) and in *A. eberthi* (HELLER, 1970a) the whole nucleus becomes incorporated into the developing gamete, only the dark, condensed area is incorporated in *E. auburnensis* (HAMMOND et al., 1969), *E. falciformis* (SCHOLTYSECK et al., 1973) and *E. maxima* (MEHLHORN, 1972b). No information on this problem is available for *E. bovis* and *E. stiedai*. Therefore, a residual part of the nucleus is formed in the above mentioned species, which is connected to the main part within the microgamete through a narrow stalk. A circular osmiophilic layer supporting the stalk is present in all species (SCHOLTYSECK et al., 1972). Finally, after the mitochondrion has been incorporated into the gamete and has assumed a position in nuclear groove, the gamete becomes free and stretches into its final, slightly curved shape. While all eimerian microgametes were found to be bounded by a single unit membrane, three membranes were reported for *A. eberthi* (HELLER, 1970a).

Of special interest is the number of flagellae in these microgametes. While 3 flagellae have been found only occasionally in *E. auburnensis* (HAMMOND

et al., 1969), this number occurs regularly in *E. falciformis* (SCHOLTYSECK et al., 1973), *E. maxima* (MEHLHORN, 1972b), *E. perforans* (SCHOLTYSECK, 1965a), and *A. eberthi* (HELLER, 1970a). Two of the flagellae, which all originate in basal bodies, are completely free, while the third one is attached for a short distance to the main body of the microgamete. In *E. maxima* (MEHLHORN, 1972b) and in *E. auburnensis* (HAMMOND et al., 1969), microtubular relics of probably a fourth flagellum were detected occasionally. In *E. maxima* (MEHLHORN, 1972), these microtubules originate from a fourth basal body. Up to now, it has been impossible to determine how the basal bodies in the tip of the microgamete develop from the 2 centrioles seen in the proximity of the nuclei during the early differentiation phase.

## Host–parasite relationship

The main place of host–parasite interaction is the parasitophorous vacuole of the host cell, which usually surrounds all intracellular stages (SCHOLTYSECK, 1963a, b; SCHOLTYSECK, 1968). The exact origin of the vacuole and of its limiting membrane is not completely understood. However, there is considerable evidence that small vesicles in the host cell cytoplasm first are arranged peripherally around the intruded parasite. Then these vesicles break and form a large vacuole containing the parasite, with the limiting membrane being built out of the membranes of the small vesicles (SCHOLTYSECK, 1963b).

On the parasite's surface, two special structures, the micropores and the intravacuolar tubules, are built up and become active during the parasite's development (SCHOLTYSECK et al., 1966a, b). In all eimerian species as well as in the genera *Babesia*, *Aggregata* and *Klossia*, the micropore consists of a bulb-like invagination of the outer pellicle membrane and a supporting collar-like opening of the inner membranes (SCHOLTYSECK and MEHLHORN, 1970). Micropores of that structure are present in motile extracellular stages like sporozoites, merozoites and microgametes as well as in several intracellular stages. However, during the phase of active growth, micropores differentiate. The invagination becomes deeper, and material from the parasitophorous vacuole is incorporated into the parasite. Then, at least in *E. tenella* (SCHOLTYSECK and STROUT, 1968), food vacuoles can be seen forming at the base of the micropore. The collar-like support ring formed by the two inner membranes is present in all stages, even in macrogamonts or schizonts, in which the inner membrane complex has been reduced in all other surface areas (SCHOLTYSECK and MEHLHORN, 1970).

The second type of organelles which can be found at the parasite's surface are the intravacuolar tubules. Up to now, we have found them in macrogametes of *A. eberthi* (HELLER, 1970a), *K. helicina* (VOLKMANN, 1967) and of all eimerian

species investigated in our laboratory except of *E. stiedai* (SCHOLTYSECK et al., 1971b). In *K. helicina*, they were found in microgametes, too (SCHULTE, 1971b). The tubules show a cross-striation in longitudinal sections and reveal, in cross-sections, filamentous subunits building up the tubule wall (found only in *E. maxima*) (MEHLHORN, 1972b). The way these tubules are attached to the parasite is not quite clear yet; they seem to originate from osmiophilic plaques immediately underneath the pellicle (SCHOLTYSECK et al., 1966a, b). In *E. perforans* (SCHOLTYSECK and SCHÄFER, 1963), these tubules touch the membrane of the parasitophorous vacuole, suggesting a role in the transport of nutrition.

In macrogamonts of *E. auburnensis* (HAMMOND et al., 1967b) and of *A. eberthi* (HELLER, 1969), deep pellicular infoldings can be observed, which differ distinctly from micropores. The pellicle, limiting these invaginations, is not changed in its ultrastructure. Occasionally, vacuoles can be seen formed at the bottom of the invaginations, suggesting a pinocytic activity.

Host–parasite relationships are not evident only in the above mentioned structures of parasitic origin, but in structures of host origin as well. The membrane of parasitophorous vacuoles harboring macrogametes often forms intravacuolar folds in many different eimerian species. Although the size of these folds varies with the species and with the developmental stage of the parasite, their width usually is about 1/3 to 1/4 of that of an intravacuolar tubule. In *E. auburnensis* (HAMMOND et al., 1967), these folds become detached from the membrane and seem to degenerate in the parasitophorous vacuole, thus increasing the amount of nutritive material available for the parasite. Similar processes occur at intravacuolar folds of the parasitophorous vacuole in macrogametes (SCHOLTYSECK et al., 1971b) and young cyst forms (MÜLLER et al., 1973c) of *Toxoplasma gondii*.

In microgamonts of *E. auburnensis* (HAMMOND et al., 1967), in macrogametes of *A. eberthi* (HELLER 1969), and in cysts of *Besnoitia jellisoni* (MÜLLER et al., 1973c), the membrane of the parasitophorous vacuole forms small vesicles which are pinched off into the vacuole, where they disintegrate.

Of special interest for evaluating the host–parasite relationship were some findings on the development of *E. tenella* in macrophages (SCHOLTYSECK et al., 1969). Although the exact developmental process involved here is not quite understood, it seems likely that schizogony (merogony) takes place in these macrophages. At the same time, degenerated (digested) merozoites and other developmental stages are present in the same macrophage. This can be interpreted as evidence for the extremely high degree of adaptation and resistance coccidian parasites can develop. Successful parasitism of a macrophage has to be considered as a major advancement.

## Cytochemistry

Cytochemical investigations have been carried out on macrogametes of *E. perforans* (SCHOLTYSECK, 1964), on macro- and microgamonts of *K. helicina* (SCHULTE, 1971a; SCHULTE, 1971b), and on merozoites of *Besnoitia jellisoni* (MEHLHORN and SCHOLTYSECK, 1973a; SENAUD et al., 1972), *Sarcocystis tenella* (MEHLHORN and SCHOLTYSECK, 1973b) and *Frenkelia* sp. (MEHLHORN and SCHOLTYSECK, 1973a). Their objective was to gain information on the nature of reserve polysaccharides and on different phosphatase activities. In all investigations, the polysaccharide was shown to consist of a glycogen-type component, which was named "Coccidien-Glykogen". In *E. perforans* (SCHOLTYSECK, 1964) and *K. helicina* (SCHULTE, 1971a), the polysaccharide granules were digested by α-amylase, and α- as well as ß-amylase were found effective in *B. jellisoni* (SENAUD et al., 1972). Therefore, the amylopectin character of these granules suggested by other authors is still doubtful (compare GEYER, 1973; KARLSON, 1972; RICHTER, 1969).

Acid phosphatase activities were found in merozoites of *B. jellisoni*, *S. tenella*, and *Frenkelia* sp. (MEHLHORN and SCHOLTYSECK, 1973a, b). Their localizations were in the endoplasmic reticulum, in the perinuclear space and between the mitochondrial membranes. In *S. tenella* and *Frenkelia* sp., additional acid phosphatase was found in the Golgi-adjunct (multimembraned vesicle) and between the middle and inner pellicular membrane. In macrogamonts of *K. helicina*, acid phosphatase was found in the wall-forming bodies (SCHULTE, 1971a).

Alkaline phosphatase activity in macrogametes of *K. helicina* occurred in micropores and in the peripheral cytoplasmic matrix, often in the proximity of wall-forming bodies (SCHULTE, 1971a). In microgamonts of this species, the enzyme activity was evenly distributed over the parasite.

## Proposals for future investigations

One of the results of our research of the last 10 years is the increasing awareness of questions not yet answered. As far as basic ultrastructure is concerned, the obstacles of oocyst and spore walls need to be overcome by some kind of cytochemical method to gain information on the morphology of the sporont. As soon as its structure is understood, the process of sporogony should be investigated and compared to schizogony (merogony). It is of high interest to find out whether sporogony, too, is to be considered as a modified endodyogeny.

Confirmation of earlier findings and further investigations are necessary for macro- and microgamonts. In macrogamonts, the findings on the pellicular

ultrastructure need to be confirmed for several different species, preferably in a comparative study using different fixation and embedding procedures. In microgamonts, the flagellar complex and the origin of the basal bodies need further clarification. Also, the residual nucleus observed in some species should be investigated in regard to the DNA — RNA-distribution.

The main problem, however, will likely be the cytochemistry of intracellular parasitism. This calls for enzymatic investigations on the metabolism of the host cell (compared to non-parasitized cells) and on problems like the metabolism of polysaccharides in the parasite. Enzymatic investigations should be carried out as well on the parasitophorous vacuole to gain information on the role of intravacuolar tubules and similar structures. Cytochemical approaches will also lead to a better understanding of motility and penetration processes. For all these investigations, tissue culture work will be extremely helpful.

Finally, the research objects for all investigations should be not only *Eimeria* spp. (with a few exceptions in the past), but should come from as many coccidian families as possible, including *Haemosporina* and *Piroplasmasida*.

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Address of the authors: Bodo MÜLLER, Dr. Erich SCHOLTYSECK, Zool. Institut, 53 Bonn 1, W. Germany

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# INFECTIOUS (VIRAL) ENTERITIS, A NEW GOSLING DISEASE IN THE SOVIET UNION

## (Review)

By

L. M. KONTRIMAVICHUS

All-Union Veterinary Research Institute (director: J. R. KOVALENKO), Moscow, USSR

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The establishment of large-scale goose farms was begun in different areas of the Soviet Union after 1960. In several farms, production results were poor and rearing losses were high. This was initially attributed to lack of experience, and, chiefly, to management failures and feeding errors. From 1963, the suspicion was increasingly gaining ground that in certain districts the losses were due to a previously unknown goose disease, which had not yet been described. Studies were immediately begun on epizootological course, symptoms and gross lesions of the disease and toxicological, bacteriological and virological examinations were performed. The causal agent was identified as a virus and the picture was named after the most characteristic symptom as "infectious enteritis" (KONTRIMAVICHUS et al., 1966).

*Epizootological observations.* Goslings simultaneously released from the hatchery usually became diseased in all farms to which they had been distributed. Many observations suggested that the causal agent is transmitted through the egg, and day-old goslings are already infected at transport from the hatchery. The average latency period was 5—10 days, but also older goslings developed the disease. Adult geese and young chicks and ducklings kept together with sick goslings did not become infected. But the disease spread to small farms in nearby villages, uniformly affecting goslings originating from the hatchery and those naturally hatched on the spot (KONTRIMAVICHUS, 1972a).

Losses usually ranged from 30 to 80%, but occasionally all goslings died. Mortality from the disease was especially high among the offspring of one-year-old layers. The outbreaks occurred among the Gorky and Kuban breeds, known for their high egg production. Day-old goslings of the Arsamas and Grey Steppe breeds could be infected experimentally, but the losses were much lower than among the other two breeds (KONTRIMAVICHUS, 1972a).

*Symptoms.* At the beginning of the outbreak, varying periods of latency were followed by an abrupt death, without preceding symptoms. Later the

disease took a longer course. The goslings showed listlessness and inappetence and huddled together, obviously seeking warmth. Some birds shook frequently their head, as if to get rid of nasal discharge, others showed a watery salivation. The body temperature of the diseased birds was 41.0—41.7 °C, not differing from that of the healthy ones. Severely diseased birds often had a watery diarrhoea, with occasionally sanguinolent streaks. Some goslings showed a protracted course of disease; these ate little, preferred to squat with the head bent to one side, and were markedly retarded. In three farms many sick goslings also showed nervous symptoms (convulsions, movement disturbances) at 2—3 weeks of age. A greater or lesser number of the sick birds recovered and could practically overcome growth retardation until about two months of age (KONTRIMAVICHUS, 1970c, 1971; KONTRIMAVICHUS and ELBERT, 1968).

*Gross lesions.* Those goslings which died abruptly in the early stage of the outbreak did not usually show gross lesions. Later the main change was enteritis, varying in type and severity. In acute cases, the enteritis was haemorrhagic, in chronic cases it was fibrinous. The fibrin sometimes formed a loosely attaching coat on the intestinal mucosa, in other cases it formed a lamellar, tubular or plug-like structure. Dense, viscous mucus was often found in the nasal cavity and sometimes also the proventricular mucous membrane was covered by a mucinous coat. Different degrees of myocardial degeneration were found; sometimes the heart was whitish and dilated. The liver was swollen and hyperaemic in most cases. In cases free from a secondary bacterial complication, the spleen was normal.

*Microscopic lesions.* Goslings died or killed in premortal state after natural or experimental infection uniformly had enteritis and often also a haemorrhagic or fibrinous enterocolitis.

The liver and kidneys were hyperaemic and groups of cells showed different degrees of degeneration. The myocardial cells also showed different stages of degeneration, up to complete disintegration.

Apart from the above changes, AKULOV (1970) observed congestion of the splenic sinusoids, proliferation of lymph follicles, increased heterophil leucocyte counts and oedema of the perivascular connective tissue and trabeculae. VERTINSKIY et al. (1973) also found lesions in the respiratory tract and in all parts of the brain.

*Chemical and toxicological examination.* No toxic levels of NaCl, nitrite, mercury or arsenic compounds were found either in the gosling feed or in gastro-intestinal contents.

*Mycological examinations.* Pathogenic fungi *(Aspergillus sp., Candida sp., etc.)* were not found in the gosling feed, nor in various organs of the carcases.

*Bacteriological examinations.* The organs of most carcases were found to be bacteriologically sterile. In certain cases, especially in the second part of the endemic outbreaks, *S. typhimurium*, *S. anatum* or *E. coli*, in other cases diplococci and streptococci were isolated from the carcases. Neither the diplo nor the streptococci were pathogenic for mice and rabbits by the intraperitoneal route. Infrequently *Clostridium perfringens* type A strains were isolated from the liver.

As to the intestinal flora, the absolute numbers of *E. coli*, *B. proteus*, *Pseudomonas sp.*, *Cl. perfringens*, and occasionally, also of *Salmonella sp.* or other facultatively pathogenic bacteria increased considerably compared to normal. The altered bacterial flora may aggravate the course of the disease and thus increase losses. At all events, the bacterium-free filtrate of intestinal contents with changed flora composition did not kill mice, rabbits and guinea pigs on intravenous and intraperitoneal administration, whence the lethal effect of bacterial toxins absorbed from the intestine could be excluded (KONTRIMAVICHUS et al., 1972).

To obtain evidence of egg transmission of the agent, the 379 goose embryos which died in different stages of hatching were also examined bacteriologically. *E. coli*, *Salmonella sp.*, *Pseudomonas sp.* and cocci were isolated from many embryos, but infection experiments in goose embryos, with each isolate in itself and in various combinations, failed to contribute any important data to the knowledge of the aetiology.

*Virological examinations.* Goslings originating from unaffected flocks were inoculated with organ homogenates or intestinal contents from carcases of goslings spontaneously died or killed in a severe state of illness. The goslings inoculated either orally, subcutaneously or intranasally developed disease. Experimental infection was also successful with Seitz filtrates of the infectious material. Out of a total of 247 goslings used in 18 infection experiments, 209 died within 12 days after infection, including latency for 4—8 days. The 172 birds set up as controls and kept in isolation from the rest did not develop the disease (KONTRIMAVICHUS, 1970a).

Isolation of the virus was initially attempted in embryonated goose eggs preincubated for 12—15 days. Certain organ homogenates killed the embryos within 2—6 days. Several embryos showed cutaneous hyperaemia and oedema, sometimes haemorrhages and liver degeneration. Later well-reproducible results were obtained in eggs preincubated for 10 days, as proposed by DERZSY et al. (1970). In the first passage only a single strain was capable

of killing all embryos. After a few transfers, however, further strains caused regularly embryonic death. The latency period became shorter, ranging from 2 to 6 days, and virus titres of the chorioallantoic fluids rose to the level of $10^{-4}$—$10^{-6}$. Apart from the lesions described in the foregoing, oedema of the chorioallantoic membrane, dilatation of the heart and myocardial degeneration were observed (KONTRIMAVICHUS, 1971).

The pathogenicity of the virus for day-old goslings did not change after five passages in primary embryonic goose kidney cell culture. Cytopathic changes began to appear from the second passage on. Rounding, vacuolization and gradual pycnosis of part of the cells was followed by their detachment from the tube wall, resulting in discontinuity of the cell monolayer (KONTRIMAVICHUS and MAYBORODA, 1968).

The virus could not be propagated in chick or duck embryos and it was not pathogenic for day-old chicks, ducks, mice, guinea pigs and rabbits. It was found to agglutinate the red blood cells of goose, duck, chicken, horse, sheep, swine, rabbit and guinea-pig (KONTRIMAVICHUS, 1971).

The virus is resistant to ether and chloroform, it is not affected by antibiotics and maintains infectivity in 40% glycerol at 2—4 °C for two years, in dried form for one year at the least. It resists heat treatment at 60° for 15 min, but is killed by on exposure at this temperature for 30 min (KONTRIMAVICHUS, 1970b, 1971).

*Diagnostic differentiation.* Although the virus responsible for the gosling disease was not pathogenic for chicks and ducks either by natural contact or by experimental infection, it seemed worthwhile to examine whether goslings were susceptible to the Newcastle disease virus (NDV) strains occurring in the Soviet Union, and to duckling hepatitis virus.

Goslings 18—26 days old were infected with the velogenic T strain of NDV, and six weeks old goslings were immunized with the mesogenic H strain. The experimentally infected birds developed nervous symptoms (paralysis of wing, torticollis, compulsive movements) and if the disease took a longer course, haemagglutination inhibiting (HI) antibodies appeared in the blood serum. The birds immunized with strain H also had serum HI antibodies up to a titre level of 1 : 320.

Experimental infection with the NDV strains T, H and LaSota was uniformly lethal to goose embryos. The dead embryos showed cutaneous hyperaemia and haemorrhages, and the chorioallantoic fluids agglutinated chicken erythrocytes up to dilutions from 1 : 640 to 1 : 2560.

It follows that young goslings are susceptible to the velogenic strains of NDV, but develop symptoms distinct from those of infectious enteritis. Infection or immunization with the vaccine strain induces an HI antibody response and the chorioallantoic fluids of embryonated goose eggs infected

with velogenic, mesogenic or lentogenic NDV strains agglutinate the chicken erythrocytes (KONTRIMAVICHUS, 1972b).

It was reported (WACHNIK and NOVACKI, 1962) that duckling hepatitis virus is pathogenic for goslings. In our own experiments, goslings infected with strains O, P or Ki of duckling hepatitis virus became diseased, their growth was retarded and 25% of them died. At *post mortem* examination, enlargement and light yellow discoloration of the liver and congestion of the gallbladder were found, but no other changes could be observed (KONTRIMAVICHUS and MAYBORODA, 1970; AKULOV et al., 1972).

The susceptibility of goose enbryos and day-old goslings to duckling hepatitis virus is an important experimental observation, but there is no evidence of its natural incidence in the Soviet Union. The symptoms showed on experimental infection were, nevertheless, distinct from those of natural and experimental infectious enteritis among goslings.

Certain authors reported susceptibility of goslings to duck plague virus, but the picture and the properties of the agent were distinct from those observed with the gosling disease.

During the recent decade a gosling disease, more or less similar to infectious enteritis, had been reported from several countries under various names, such as "goose influenza", "goose plague", "ascitic hepato-nephritis", "gosling hepatitis", *etc.* From the diseased or dead birds chloroform-resistant virus strains were isolated, which were classified by some workers as reoviruses, by others as parvoviruses. Two authors (CSONTOS, 1967; KALETA, 1969) reported isolation of adenovirus strains from droppings of healthy geese and from liver homogenates of diseased geese, but did not comment on pathogenicity. The goose adenovirus isolate of COUDERT (personal communication) was not pathogenic for goslings.

It follows that none of the known goose viruses can be held responsible for the disease causing outbreaks in the Soviet Union since the years 1960—1963. The causal agent of the disease termed as "infectious enteritis" is clearly an as yet unknown virus whose systematic position remains to be clarified like that of the strains isolated from the gosling disease in other countries.

*Therapy and prevention.* Treatment with different antibiotics, chemotherapeutics and paratyphoid serum, either alone or in various combination, had neither therapeutic, nor preventive effects (KONTRIMAVICHUS et al., 1966).

Sera from convalescent geese contain antibodies and confer a high-degree protection on susceptible goslings at an 0.5—1.5 ml prophylactic dose level. Losses among goslings preventively treated with convalescent serum ranged from 0 to 14%, compared to a 50—100% loss in the control groups, which were given paratyphoid antiserum, normal goose serum, antibiotic, or no treatment whatever (KONTRIMAVICHUS et al., 1966; KONTRIMAVICHUS,

1968, 1969, 1971). According to KAPITANAKI and CHECHULA, (1970) and FADYIN et al. (1971), the citrated blood of convalescent geese is of similar protective effec tto convalescent serum.

Veterinary hygienic and administrative measures are now regularly taken to prevent the spread of the gosling disease. Introduction of geese from infected farms to "clean" flocks is prohibited. Infected flocks are treated as closed stocks and restrictions are imposed upon sale of eggs and geese. Regular disinfection of goose houses, hatcheries and machinery is performed (KONTRIMAVICHUS, 1971). These measures seem to be sufficient for the prevention of further spread. The duration of carriership is not yet known and for the time being regular treatment with convalescent serum or blood is the only means of reducing losses.

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Address of the author: Dr. L. M. KONTRIMAVICHUS, Vsesojuznij Inst. Experimentalnoj Veterinarii, 109472 Moskva Kuzminki, UdSSR.

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# EXPERIMENTAL INFECTION OF LAMBS WITH REOVIRUS TYPE 1 ISOLATED FROM SHEEP

By

S. BELÁK and V. PÁLFI

Department of Epizootiology (Head: Prof. J. MÉSZÁROS), University of Veterinary Science and the Central Veterinary Institute (Head: A. VÁNYI), Budapest

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A respiratory and intestinal disease of lambs (BELÁK et al, 1974) and the partial characterization of the isolated virus (BELÁK and PÁLFI, 1974) were reported previously.

Results of the experimental infection of lambs with the isolated reovirus designated H/11 are presented in this paper.

## Materials and Methods

Seven artificially nursed, 2—4 weeks old merino lambs were experimentally inoculated. Blood samples were examined for antibodies, basal temperature was recorded, nasal, conjunctival and rectal swabs were collected prior to infection. The virus for inoculation was in its 3rd passage and propagated as described previously (BELÁK and PÁLFI, 1974). Supernatants of infected and control cell cultures were centrifuged at 5000 r.p.m. for 30 min at 4 °C. The media were tested for mycoplasmas. Five lambs were inoculated intratracheally with 1 ml, and intranasally with 2 ml of a suspension containing $10^3$ $TCID_{50}$/ml H/11 virus. The two control lambs were similarly inoculated with the supernatant of uninfected cell cultures. The inoculation was repeated on the following day. Nasal, conjunctival and rectal swabs were collected daily for 3 weeks and rectal temperatures recorded twice a day. Swabs were examined for the presence of virus as described in detail previously (BELÁK and PÁLFI, 1974). One infected lamb was killed on day 8 post infection (p.i.). Its organs and those of another died on day 11 p.i. were examined pathologically, virologically and for the presence of bacteria, mycoplasmas and chlamidiae. Second blood samples were collected from all the survivors on day 21 p.i. Both first and second blood samples were examined for the presence of virus-neutralizing antibodies. Paraffin-embedded and haemalaun–eosin stained preparations were examined histopathologically.

## Results

The first clinical symptoms appeared between day 4 and 6 p.i. The animals showed a temperature rise of 0.6—0.9°C. Four lambs had diarrhoea, their condition worsened on the second week. Serous ocular and nasal discharge, sneezing and dyspnoea could be observed in all infected lambs. Mild enteritis, enlarged intestinal and peribronchial lymph nodes, rhinitis, tracheitis and consolidation of lungs were found macroscopically in the lamb killed 8 days p.i. Microscopically a mild catarrhal inflammation with a beginning necrosis of the upper layer of mucosa (Fig. 1) were seen in some parts of the nose and a slight proliferation on other areas (Fig. 2). The mucous membrane was infiltrated by lymphocytes and granulocytes. Infiltration was stronger in some submucous areas. Diffuse interstitial pneumonia was found in the lungs, the interalveolar septa were thickened due to mononuclear infiltration, the lumen of alveoli and ducti alveoli collapsed (Figs 3, 4). In the lumen of several alveoli desquamated and degenerated alveolar epithelial cells were seen (Fig. 5). Several inflammation cells also appeared among these epithelial cells.

One of the lambs died on the 11th day p.i. Its death was preceded by a temperature rise of 1.2 to 1.4 °C. Enteritis, a focal catarrhal pneumonia in the anterior parts of the lungs (Fig. 6) and a catarrh of the upper airways were found pathologically. Bacteriological examination of the spleen, lungs, peribronchial and intestinal lymph nodes of killed and died animals gave negative results. Neither mycoplasmas nor chlamidiae could be isolated from

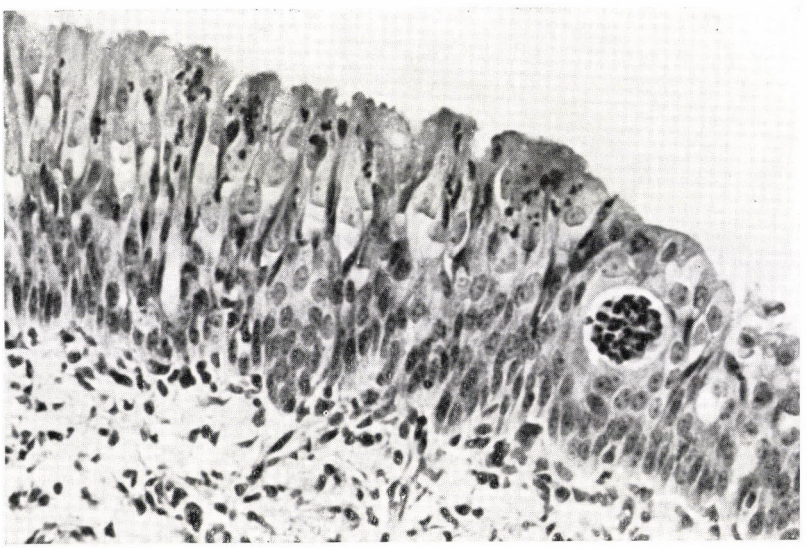

*Fig. 1.* Mucosa in the nose of a 3 weeks old lamb with a mild catarrhal inflammation, HE stain, ×400

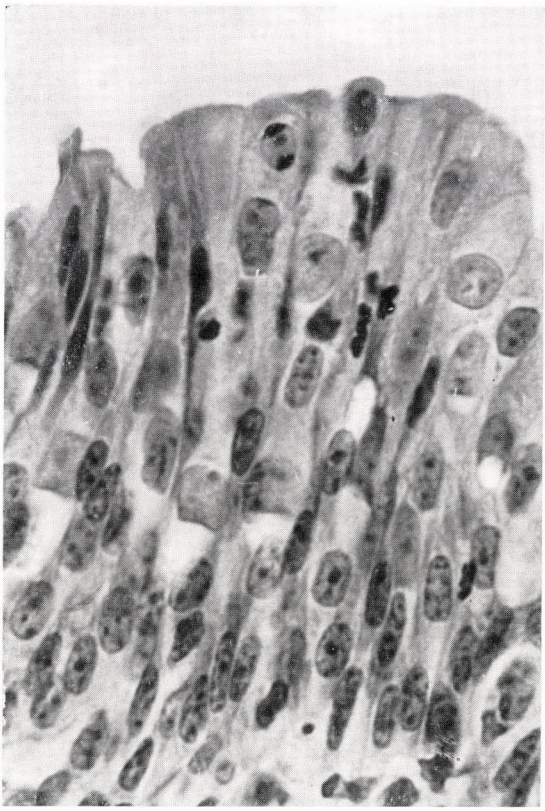

*Fig. 2.* Mucosa with a slight proliferation in the nose of a lamb aged 3 weeks, HE stain, ×650



*Fig. 3.* Interstitial pneumonia in a 3 weeks old lamb. Infiltration of interalveolar septa, HE stain, ×95

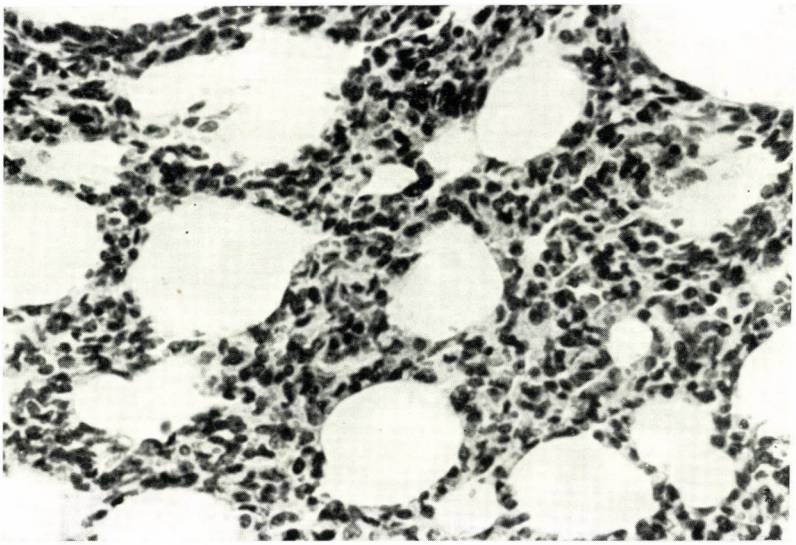

*Fig. 4.* The interalveolar septa infiltrated with inflammation cells in the lungs of a 3 weeks old lamb, HE stain, ×400

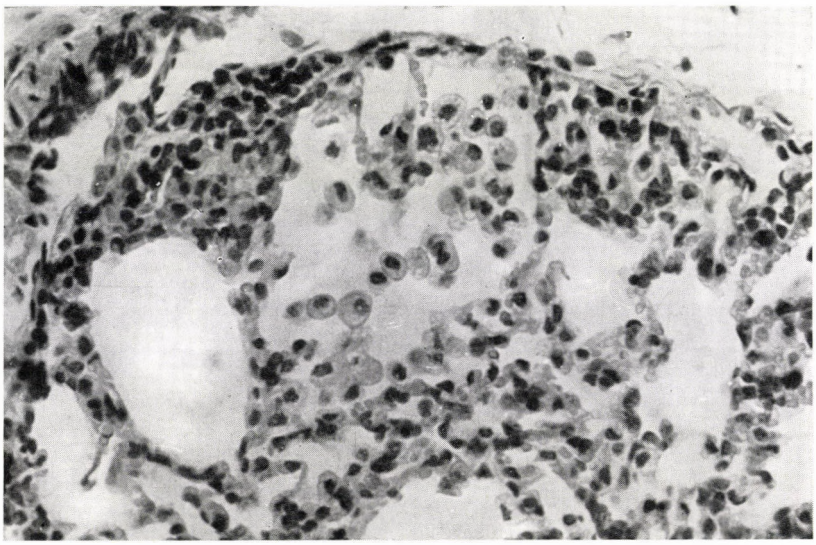

*Fig. 5.* Desquamated and degenerated alveolar epithelial cells in the lumen of alveoli, HE stain, ×400

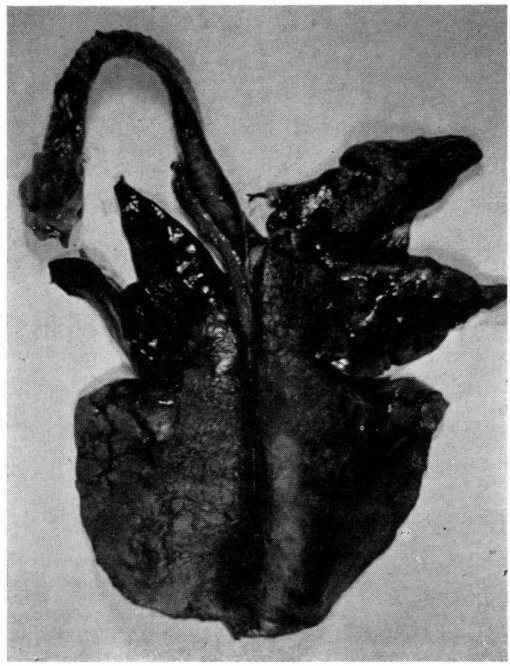

*Fig. 6.* Lungs of a 3 weeks old lamb, focal catarrhal inflammation

the lungs. Enterococci and non-haemolyzing *E. coli* were cultivated from the intestines. The further lambs of the infected group showed the mild respiratory and intestinal symptoms as described above. These clinical symptoms lasted approximately for two weeks.

On the 4 to 9th day p.i. the H/11 strain could be re-isolated from the nasal and rectal swabs collected from the infected animals. Virus was not isolated from the conjunctivae and from the suspensions prepared from the organs of killed and died animals. No specific antibodies were found in the blood of the lamb killed on day 8 p.i. In the second blood samples collected 21 days p.i. neutralizing antibodies appeared at a titre of 1 : 64 in two, and 1 : 128 in one animal.

## Discussion

Although the H/11 virus strain was isolated from an epidemic in which a viral background was supposed, and where a titre rise in specific antibodies was found, it seemed to be necessary to investigate the pathogenicity of the isolate. In spite of the wide distribution of reoviruses there are different opin-

ions concerning the role of these agents in inducing disease (BASKERVILLE et al., 1971; LAMONT, 1968; MCFERRAN and CONNOR, 1970; PHILIP et al., 1968; PHILIP, 1970; ROSEN and ABINANTI, 1960). Both successful and unsuccessful attempts to produce experimental disease have been reported.

LAMONT et al. (1968) reported on experimental infection of calves with type 1 and type 2 reoviruses. They found that reoviruses were involved in respiratory diseases of this species. Reovirus type 3 has been isolated by SCOTT et al. (1970), from a cat supposed to be died of panleucopenia. In experimental infection reoviruses were found to be of mild pathogenicity to cats. In a similar experiment interstitial pneumonia could be produced in dogs (LOU and WENNER, 1963) experimentally infected with reovirus. Field cases and experimental diseases were observed in mice and rats (COOK, 1963; KILHAM and MARGOLIS, 1973; PHILLIPS et al., 1970). Reovirus has also been isolated from swine (KASZA, 1970). In the case of this species further investigations are needed to clarify the pathogenicity of reoviruses.

The first isolation of reovirus from sheep has been reported recently (MCFERRAN et al., 1969; MCFERRAN et al., 1973). The strains, isolated in Northern Ireland from the faeces of both clinically normal and diseased lambs, all belonged to type 3 of reoviruses. Until now no reports have been available on isolation of other types of reovirus from sheep or on the pathogenicity of reoviruses in this species.

On the basis of the results presented here it can be stated that the first type 1 reovirus strain, isolated from an epidemic en masse and designated H/11, was capable of causing disease in young lambs after an incubation period of 4 to 6 days. The upper and lower respiratory and intestinal tracts were involved in the experimentally induced disease and similar clinical signs were observed in the field cases. Bacteria could not be cultivated from the diseased and other parenchymal organs, a normal intestinal bacterial flora was found. The animals shed the virus with their nasal discharge and faeces and gave serological response.

The seriousness of the symptoms was influenced to a high extent by the individual resistance of infected animals. Lambs of high vitality showed relatively mild clinical symptoms. The symptoms ceased at the end of the third week p.i. On the basis of field cases it is most likely that in animals of lower vitality the virus infection can be fatal without any bacterial complication. But the heavy losses recorded in the two large farms were due to virus-induced processes complicated by secondary bacterial invasion. The climatic disturbances, extremely intensive, unnatural keeping and unsatisfactory housing experienced in the field cases can make the symptoms more serious and influence the disease basically induced by the reovirus.

*Acknowledgement.* Thanks are given to E. TÚRY for preparing the histopathological preparations.

## Summary

Lambs were infected experimentally with reovirus strain type 1 (H/11) isolated from sheep during a field epidemic. After an incubation period of 4 to 6 days 0.6 to 0.9°C rise in temperature, ocular and nasal discharge, sneezing, dyspnoea and diarrhoea could be observed in 2 to 4-week-old lambs infected intratracheally and intranasally. Enteritis, catarrh of the upper airways and diffuse interstitial pneumonia were found in a lamb killed 8 days post infection (p.i.). The symptoms worsened in a lamb of lower vitality and this animal died on day 11 p.i. Necropsy revealed enteritis, catarrh of the upper respiratory tract and multifocal catarrhal pneumonia. Clinical signs ceased in the survivors at the end of the third week, and specific neutralizing antibodies in titres between 1 : 64 and 1 : 128 appeared in the convalescent sera against the H/11 strain. Virus could be reisolated from the nasal and rectal swabs between 4 and 9 days p.i. On the basis of the experimental infection and field cases it is concluded that reoviruses play an active role in the aetiology of respiratory and intestinal diseases of young lambs.

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Address of the authors: Dr. Sándor Belák, Dr. Vilmos Pálfi, 1143 Budapest, Hungária krt. 23, Hungary

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# CHARACTERIZATION OF A HERPESVIRUS ISOLATED FROM SPONTANEOUSLY DEGENERATED BOVINE KIDNEY CELL CULTURE

By

S. Belák and V. Pálfi

Department of Epizootiology (Head: Prof. J. Mészáros) of the University of Veterinary Science and the Central Veterinary Institute (Head: A. Ványi), Budapest

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Most of the bovine herpesviruses described so far were isolated from diseased animals (IBR, bovine mammilitis, pseudorabies, malignant catarrhal fever). An antigenically different group of the herpesviruses with not clarified aetiological significance was isolated from cattle showing different clinical symptoms (Bartha et al., 1967; Storz, 1968; Bodon et al., 1970; Mohanty et al., 1971; Smith et al., 1972; van der Maaten and Boothe, 1972). Luther et al. (1971) have recently isolated a herpesvirus from the kidneys of an apparently healthy calf following a 29-day-long cultivation period. Ludwig and Storz (1973) reported on the activation of a herpesvirus from normal bovine foetal spleen cells after a prolonged cultivation.

The present report describes the isolation of herpesvirus from normal calf kidney cell cultures after a relatively short cultivation period.

## Materials and Methods

Monolayers were prepared from the kidneys of a two months old calf showing no clinical signs or lesions. Primary and secondary bovine kidney cell cultures (BK) were grown in Eagle's Basal Medium supplemented with 10% and 1% foetal calf serum for growing and maintenance, respectively. Fixed cells grown in coverslip cultures were stained with haemalaun–eosin or with acridine orange and examined for viral inclusions. Electron microscopic observations, physicochemical tests and characterization of viral nucleic acid were carried out as described elsewhere (Belák and Pálfi, 1974). Plaque formation was observed in Petri dishes, using a semisolid overlay medium consisting of Parkers's Medium 199,5% foetal calf serum and agar or methylcellulose (Tylose, MH 4000, Hoechst). The cultures were kept in a humified 5% $CO_2$ incubator at 37°C for six days, fixed in formalin and stained with Giemsa stain. Antisera were prepared in rabbits, chessboard virus neutralization tests were carried out in test tubes with the following viruses and antisera: IBR, pseudorabies, bovine adenovirus type 1, equine rhinopneumonitis (ERP), bovine herpes mammilitis (BHM) and bovine herpesvirus Móvár 33/63. Two calves aged 3 months were inoculated intratracheally (3 ml), on

to the conjunctiva (0.2 ml) and intranasally (3 ml) by injecting of a $10^{6.0}$ $TCID_{50}$/ml suspension of the isolate in its 5th passage. Rectal temperatures were taken daily and the animals were examined clinically. Nasal swabs were collected every second day during a 2-week observation period.

## Results

Small groups of rounded and refractile cells forming plaques were observed in spontaneously degenerating secondary BK cultures after an 8-day-incubation period. Cowdry type A intranuclear inclusion bodies were detected in haemalaun–eosin-stained cells (Fig. 1). The CPE disappeared in two consecutive blind passages and could be observed again in the third passage. It appeared already on the third day post inoculation (p.i.) in the higher passages. Stained with acridine orange, infected cells contained yellow-green nuclear inclusions, characteristic of herpesviruses. Incomplete viral particles in the nucleus (Fig. 2) and membrane-limited vacuoles in the cytoplasm (Fig. 3) were seen in ultra-thin sections. In physicochemical tests the isolate designated BPX/11 proved to be sensitive to heat, chloroform, trypsin, pH 3, saponin and sodium deoxycholate and it could not be stabilized with bivalent cations. Its replication was inhibited by DNA inhibitors. It formed very small plaques

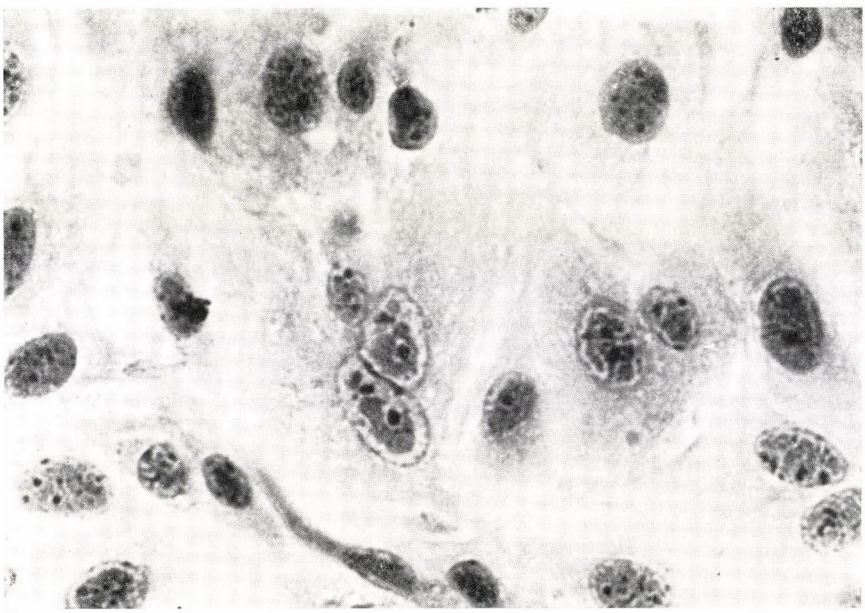

*Fig. 1.* Intranuclear inclusion bodies, 72 hours p.i. Bovine kidney cell culture. HE stain, ×300

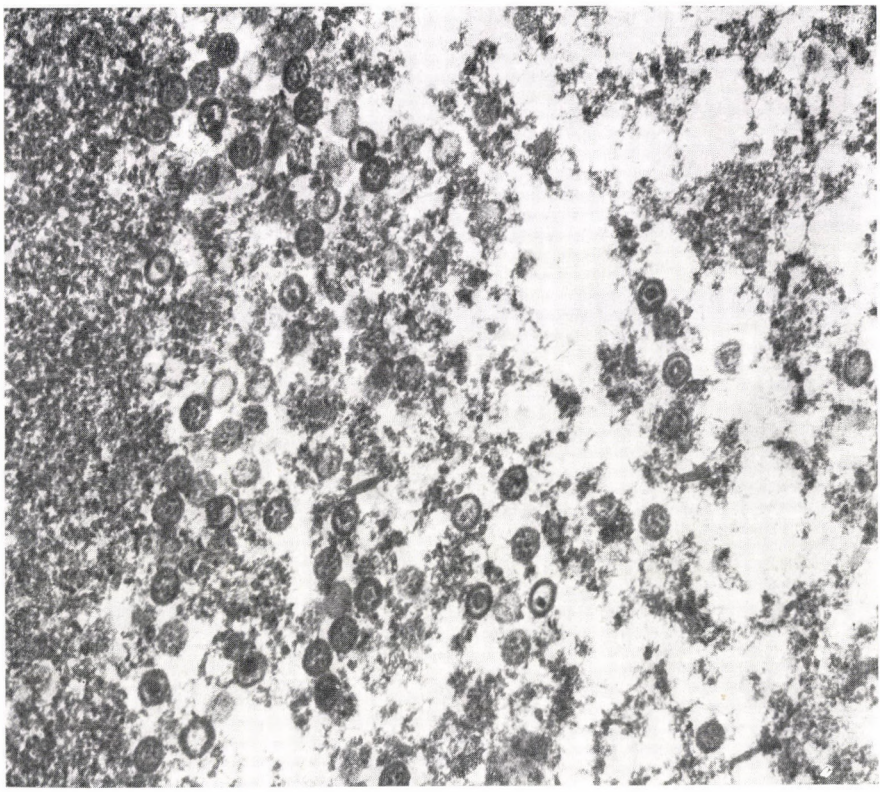

*Fig. 2.* Incomplete particles of BPX/11 strain in the nucleus of a bovine kidney cell, 72 hours p.i. Ultra-thin section, ×39,000

under the agar, and plaques 1 to 2 mm in diameter under the methylcellulose overlay. The strain showed very low antigenicity in rabbits. In serum neutralization tests it proved to be distinct from IBR, pseudorabies, bovine adenovirus type 1, ERP, BHM, but serologically related to the Móvár 33/63 strain. During the observation period the calves showed no clinical symptoms and the virus could not be reisolated.

## Discussion

On the basis of its physicochemical and antigenic properties the BPX/11 isolate proved to be different from the viruses of infectious rhinotracheitis and bovine herpes mammilitis and to belong to the bovine herpesvirus type represented by the Móvár 33/63 strain. The BPX/11 strain was isolated from cell cultures prepared from the kidneys of an apparently healthy calf. Isolation

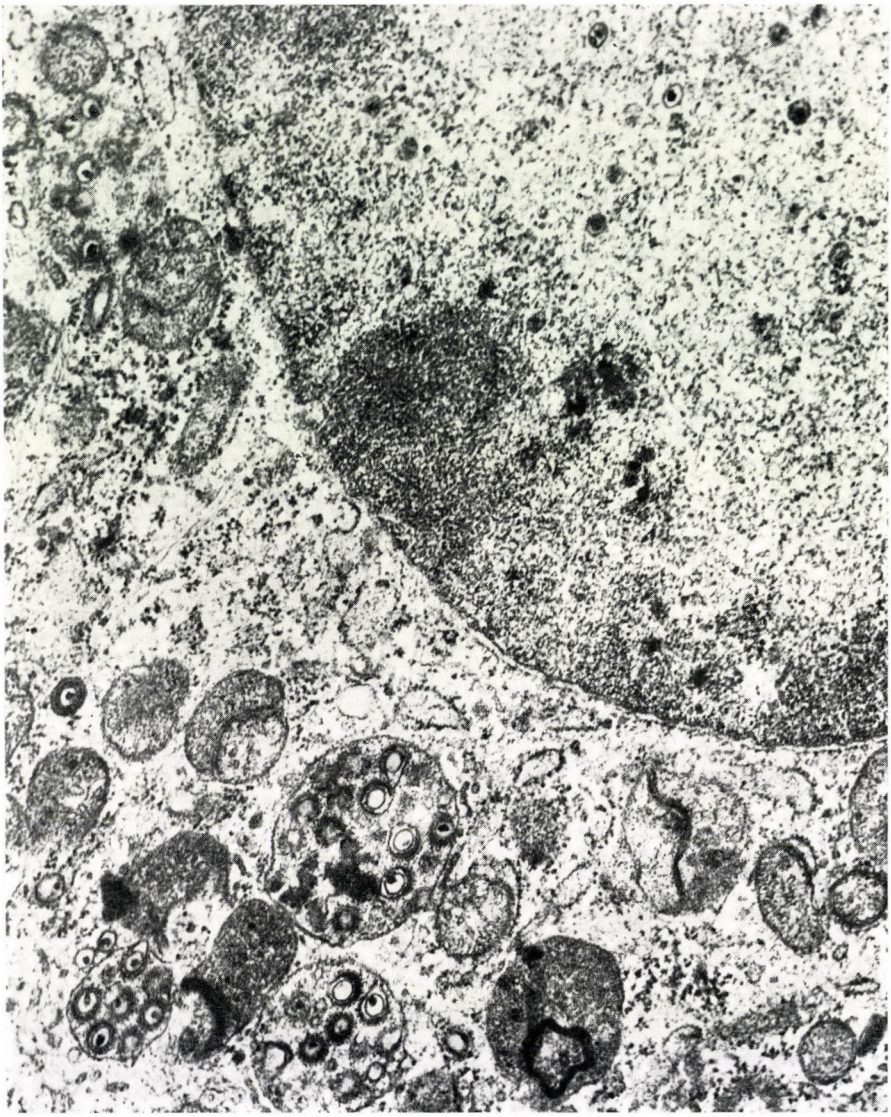

*Fig. 3.* Free incomplete particles of the BPX/11 strain in the nucleus and complete virions in vacuoles in the cytoplasm. Ultra-thin section of a bovine kidney cell 72 hours p.i. ×28,000

of a bovine herpesvirus with similar properties from an apparently healthy calf was reported only by LUTHER et al. (1971), following a 29-day-long cultivation. The BPX/11 strain produced CPE in its first passage on the 8th day, in later passages 48 to 72 hours p.i. Experiments on a limited number of animals indicated that it was not pathogenic for calves, although the con-

ditions caused by this latent virus may be different in large cattle populations. The new isolate showed low antigenicity in rabbits. These findings present further information on the presence of latent herpesviruses in apparently normal organs.

## Summary

Isolation of a virus from spontaneously degenerated tissue culture of bovine kidney origin is reported. The isolate represents a herpesvirus on the basis of its physicochemical, morphological and biological properties. Serologically it is related to the Móvár 33/63 strain of bovine herpesvirus. In experimental infection no pathogenicity was observed. The fact of isolation presents further information that apparently normal calves can carry herpesviruses.

*Acknowledgements.* The authors are indebted to Dr. P. KAPP for the electronmicroscopic observations and to Dr. A. BARTHA for his advices.

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Address of the authors: Dr. Sándor BELÁK, Dr. Vilmos PÁLFI, 1143 Budapest, Hungária krt. 23, Hungary

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# ELECTRON MICROSCOPE STUDY OF THE EARLY LESIONS IN MAREK'S DISEASE

## I. CYTOMORPHOLOGY OF THE LESION

By

H. N. Thakur

Department of Pathological Anatomy (Head: Prof. A. Kardeván)
University of Veterinary Science, Budapest

(Received January 21, 1974)

The ultrastructure of the visceral tumours and peripheral nerve lesions in field cases of Marek's disease has been described (Okada, 1970; Okada and Fujimoto, 1971; Wight, 1969), but little is known of the early lesions and their fine structure in the acute form of MD.

In the course of our study on the histogenesis of acute MD, some of the early lesions were examined with electron microscope. In the present paper the cytomorphology of the tumours and lesions is described. The results confirm our earlier observations (Kardeván et al., 1973) that the lymphoid cells, which form the tumour lesions in acute MD are cytomorphologically uniform and characteristic for the tumour cells.

### Materials and Methods

One-day-old chicks were infected intraperitoneally with a virus strain, obtained from the Veterinary Medical Research Institute, Hungarian Academy of Sciences, Budapest. The strain had been isolated from acute MD. The virus-inoculated chicks were killed 21, 26 and 33 days after infection and portions of fresh tissues showing lesions of MD were removed from heart, liver, kidney, spleen, ovary, bursa of Fabricius and peripheral nerves. For comparison, tissue fragments were collected from a freshly-killed control chick, kept separately in an isolator.

Tissue specimens were fixed in buffered osmium tetroxide (Pallade), dehydrated in ethanol and embedded in Durcupan. Ultra-thin sections were cut with Reichert ultramicrotome and electron micrographs were prepared with a Tesla BS type 613 electron microscope.

### Results

The visceral tumours varied in size, but in general they were small, nodular, whitish and appeared homogeneous on their cut surface. They consisted of lymphoid cells and the cytomorphology of the tumour cells in different organs was nearly the same.

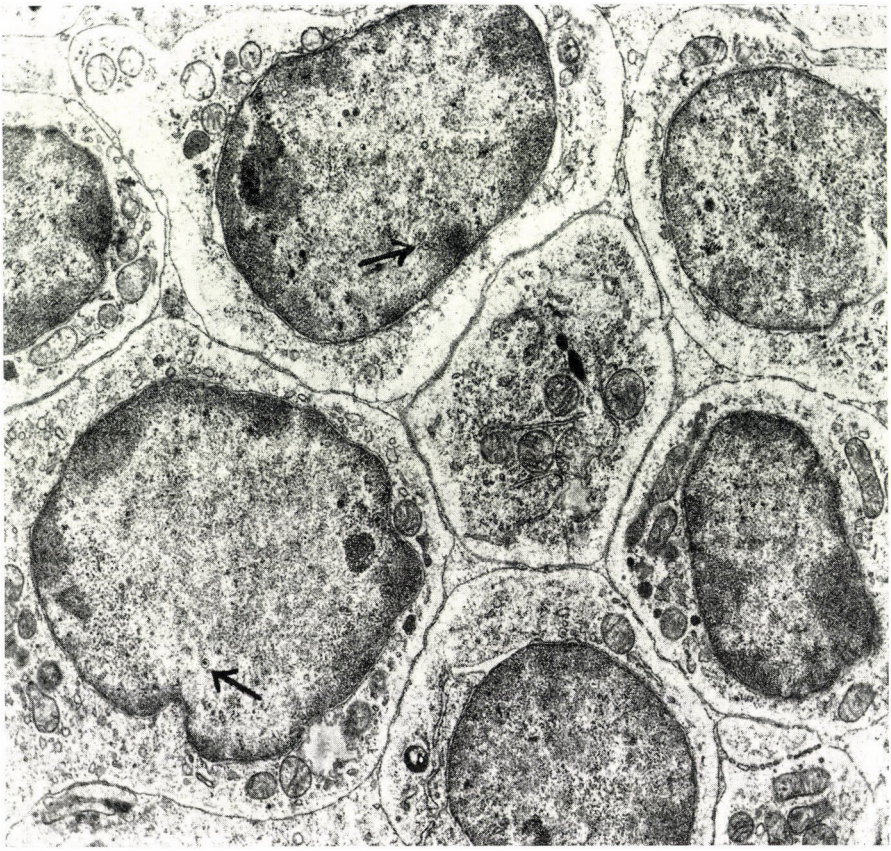

*Fig. 1.* Lymphoid cells in the ovary tumour lesion: note the intranuclear herpes virus particles (arrow) ×9260

The lymphoid cells were of variable size and large. But the general appearance of all the tumour cells was nearly the same and they differed only little in shape and morphology. In the tumours, the proliferated cells were very close to each other and only little or no intercellular substance were observed (Fig. 1).

The tumour cells were almost round or oval, sometimes slightly polygonal in shape. The hyaloplasm was less electrondense. In the cytoplasm only few mitochondria were observed, which were distributed in small groups and usually located at one pole of the tumour cell (Figs 2, 8).

Mitochondria in the young tumour cells were uniform and large, but in those showing pronounced nuclear lesions, some larger mitochondria were also observed (Fig. 6). The Golgi apparatus in the tumour cells was small

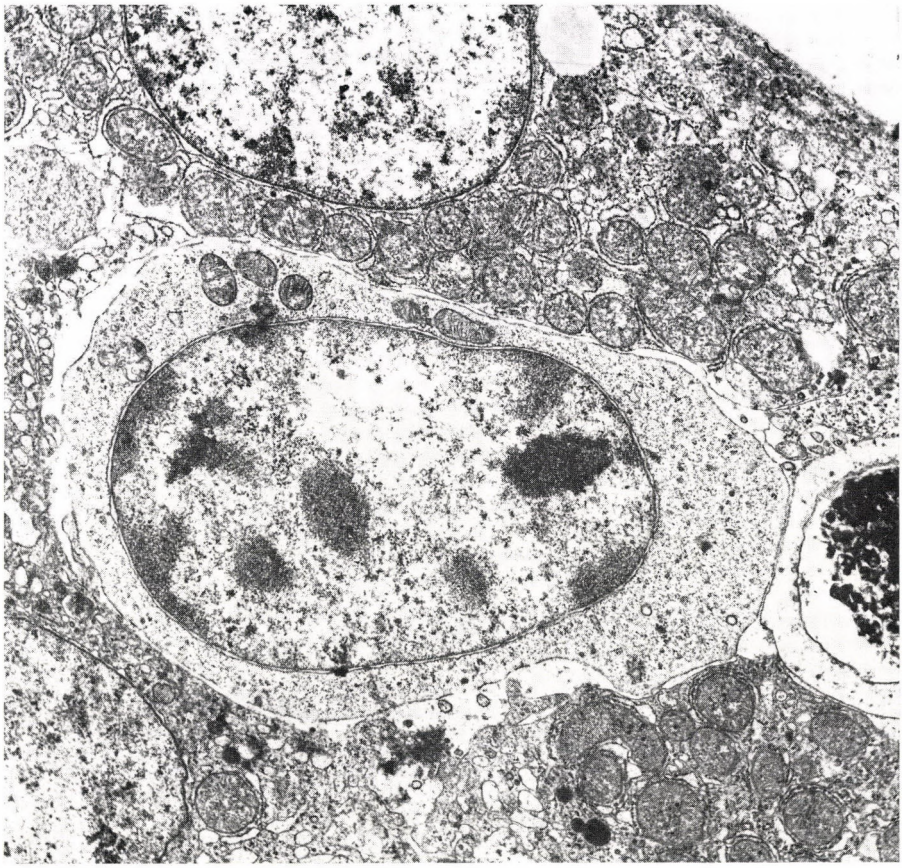

*Fig. 2.* Lymphoid cells with few mitochondria, light endoplasmic reticulum and poor cytoplasmic cell organelles in liver lesion ×9260

and poor. It was made up of only little lamellar membranes with flat vesicle (Fig. 8). The rough and smooth endoplasmic reticulum was only scattered here and there, and the greater part of the ribosomes were either free or evenly dispersed. Sometimes they were clustered in small groups (Figs 2, 8, 9).

The nucleus of the tumour cells was round or oval, and, in general, larger than the normal nucleus. The ratio of the nucleus to the cytoplasm was raised. The greater part of the nuclear chromatin was often adhered with the nuclear membrane, forming a dark clump near the nuclear border (Figs 1, 2.).

In the centre of some of the nuclei a clear zone was observed, and in the nucleus chromocentromes, remnants of the dense, compact, aggregated chromosomes were often dispersed (Fig. 2).

In other occasions, disintegration of the nuclei and nuclear membranes

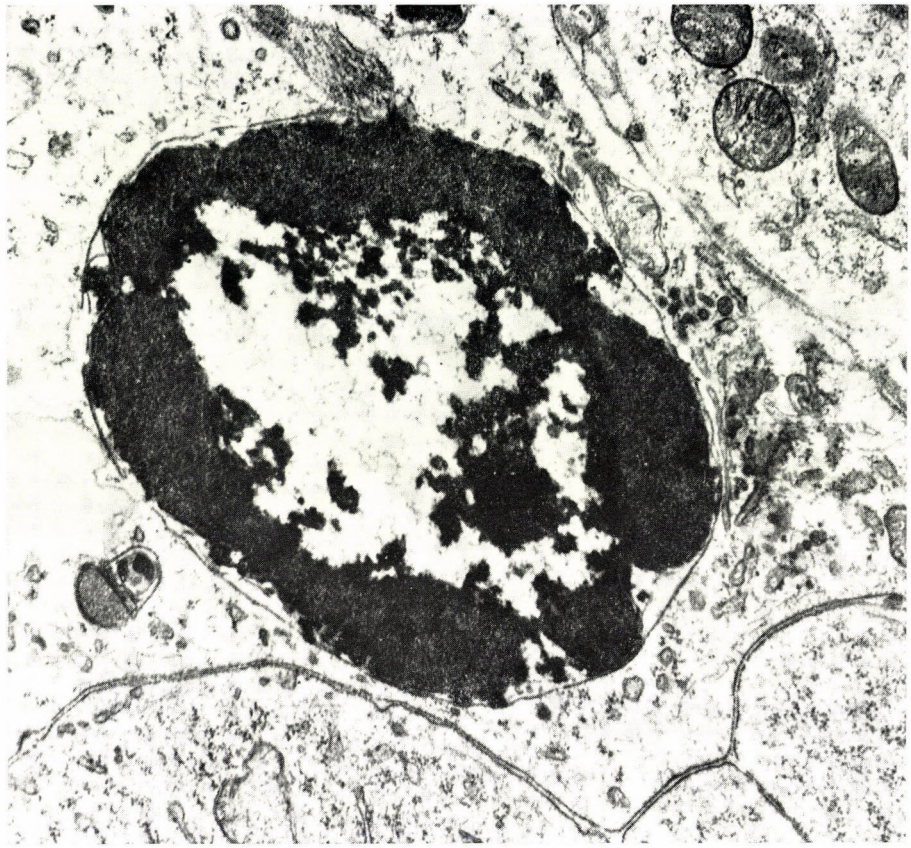

*Fig. 3.* Severe condensed dark chromatin material making a dark ring along the nuclear membrane of an ovarian tumour cell ×19,800

were observed (Fig. 4). Early signs of nuclear disintegration were often seen' with severe condensed dark chromatin material forming a dark ring along the nuclear membrane (Fig. 3).

Very often, nuclear invagination and evagination, one or several in number was observed, resulting in a deformation of the nucleus (Figs 6, 7).

The invaginated portion of the nucleus often contained cytoplasmic organelles (Fig. 5).

The nucleolus in the most of the tumour cells was large, prominent, electrondense and homogeneous (Figs 2, 4, 9).

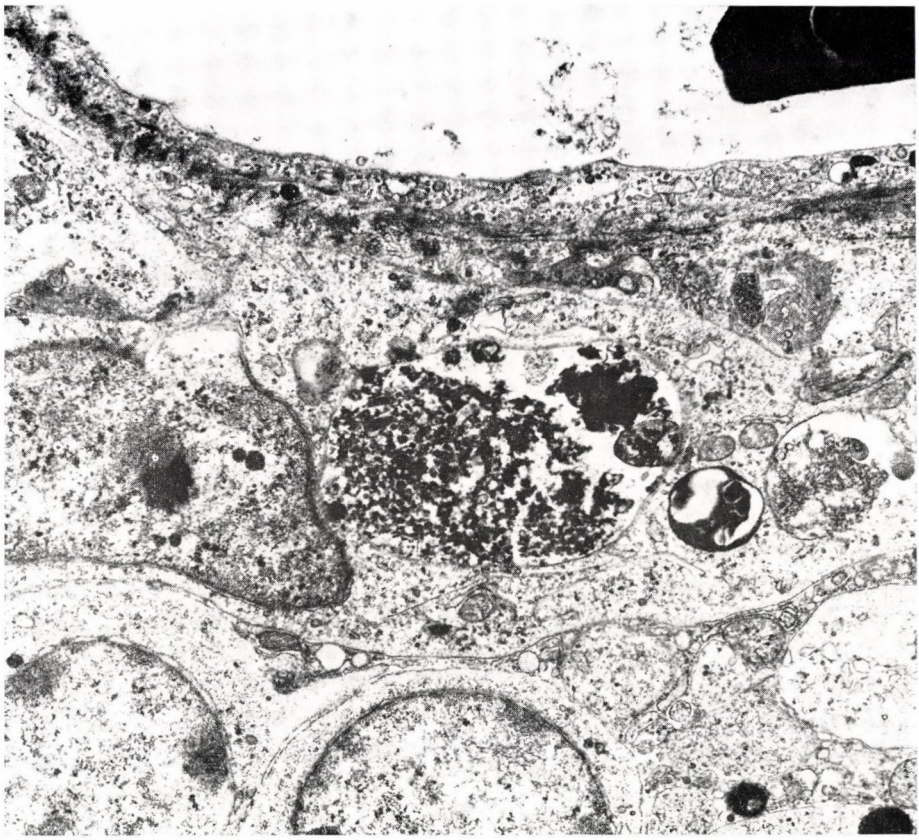

*Fig. 4.* Tumour cells in the liver showing disintegration of the nucleus and the nuclear membrane ×9260

## Discussion

The results of our investigations with light (KARDEVÁN et al., 1973) and electron microscope suggest that the lesions of the MD were formed by lymphoid cells. Similar results were published by DOAK et al. (1973), after our work had been completed and the ultrastructure of the visceral lesions in MD has been the subject of few other publications (OKADA, 1970; MLADENOV et al., 1972). Our materials for this study were collected from comparatively early lesions of experimentally produced MD. In this and the previous reports it was shown that the tumour lesions in MD were formed by lymphoid cells, immature and undifferentiated in character. The tumour cells varied significantly in morphology from normal lymphocytes. They were poor in cytoplasmic cell organelles and many of them showed nuclear invagination and evagination.

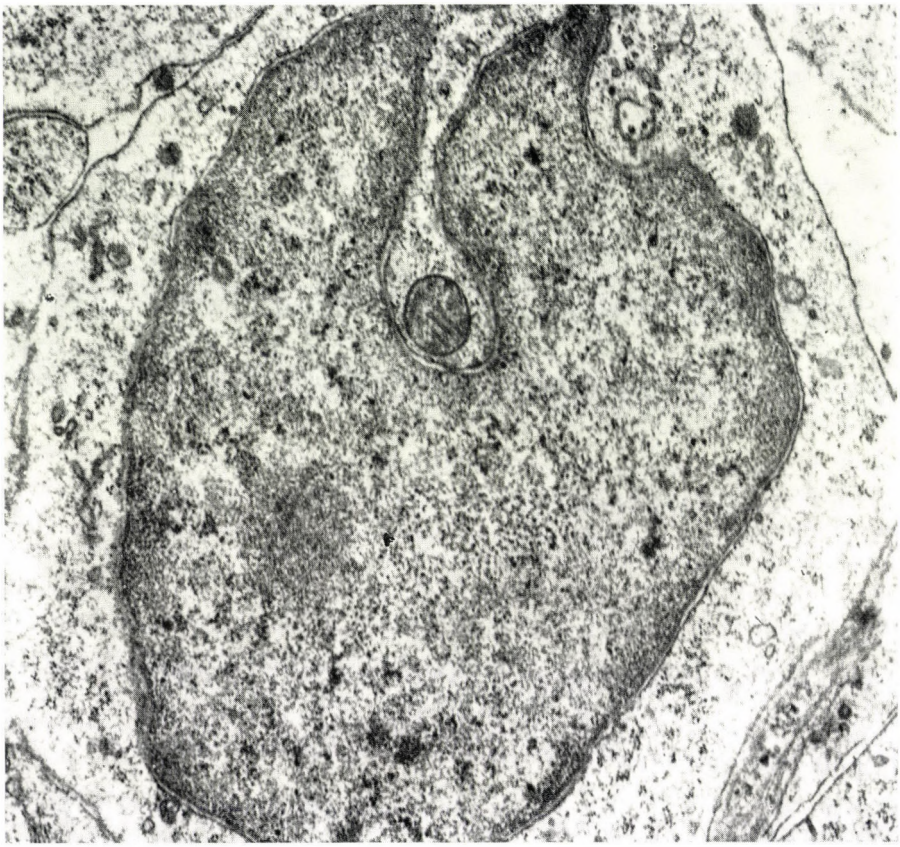

*Fig. 5.* Nucleus of a tumour cell in kidney showing invagination and pocket formation; note the cytoplasmic cell organelles ×19,800

In the present work, the ultrastructure of normal lymphocytes was not investigated separately. The ultrastructure of the tumour cells was compared with that of the normal lymphocytes as described by others (MAXWELL and TREJO, 1970). The lymphoid cells in the tumour lesions in MD were larger than the normal lymphocytes. The cytoplasm of the tumour cells was poor in cell organelles and the younger ones contained excess mitochondria in accordance with earlier findings (OKADA, 1970; MLADENOV et al., 1972). The immature lymphoid cells in MD had a morphological structure and appearence typical and characteristic of tumour cells (ACHONG and EPSTEIN, 1966; ANDERSON, 1966; MILLAR et al., 1969) with a characteristic change in the shape of the nucleus.

The chromatin substance in the tumour cells was loose and slightly dispersed. There were characteristic invaginative and evaginative changes, pock-



*Fig. 6.* Nucleus of an ovarian tumour cell with several invaginations, evaginations and unipolar mitochondria ×19,800

et formation and similar other changes in the form and shape of the nucleus. However, some of the other changes in the morphology of the nucleus, viz. nuclear blebs, ear protrusion and multivesicular nuclear formations described in spontaneous cases of MD (MLADENOV et al., 1972) were not observed in the early lesions.

In one case intranuclear herpesvirus particles were observed in the ovary tumour lesions of MD (KARDEVÁN et al., 1973; THAKUR, 1973). These virus particles were naked and incomplete (Fig. 10).

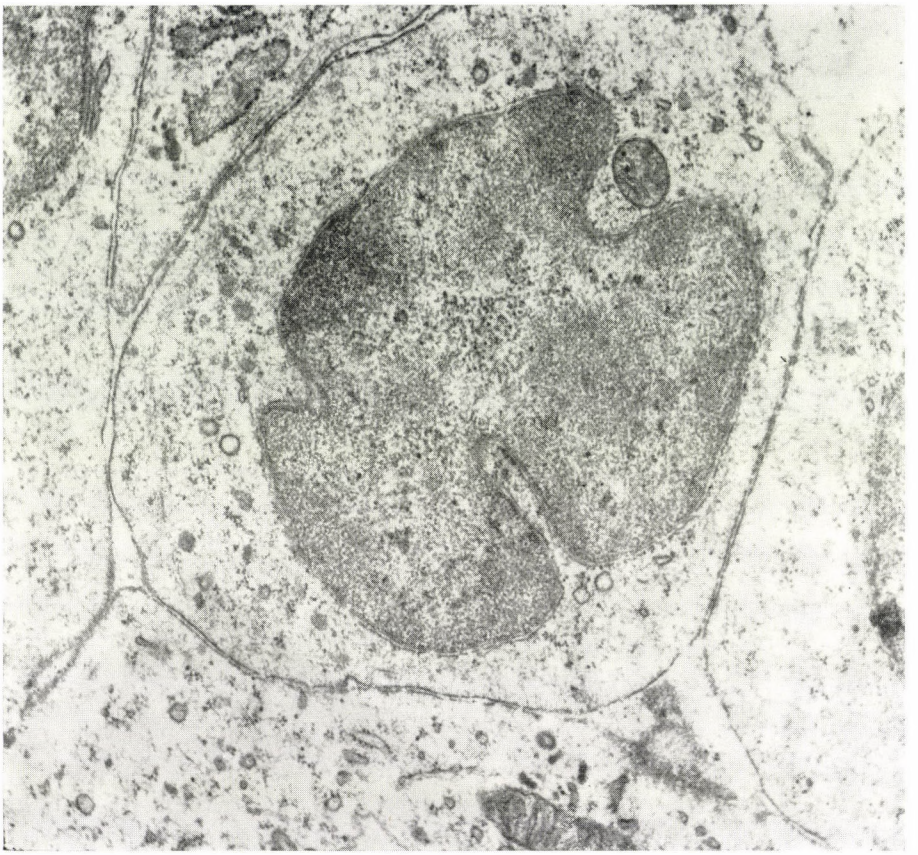

*Fig. 7.* Tumour cell in the kidney showing nuclear invaginations and poor cytoplasmic cell organelles ×19,800

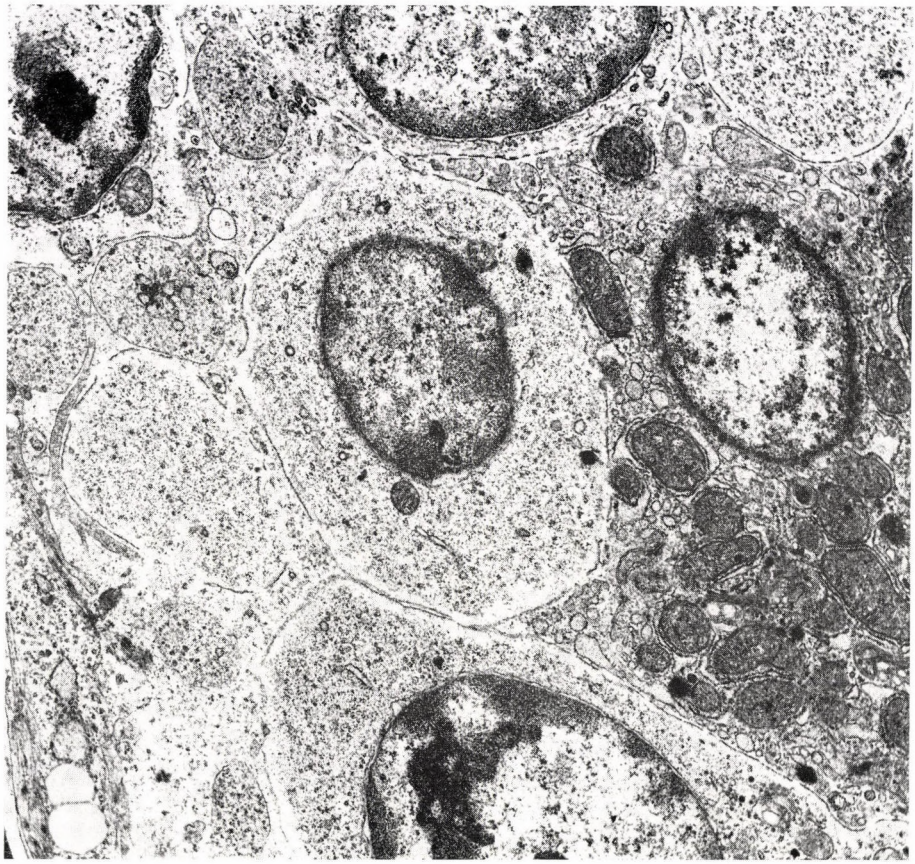

*Fig. 8.* Tumour cell in the liver showing poor Golgi apparatus, little lamellar membrane with flat vesicle ×9260

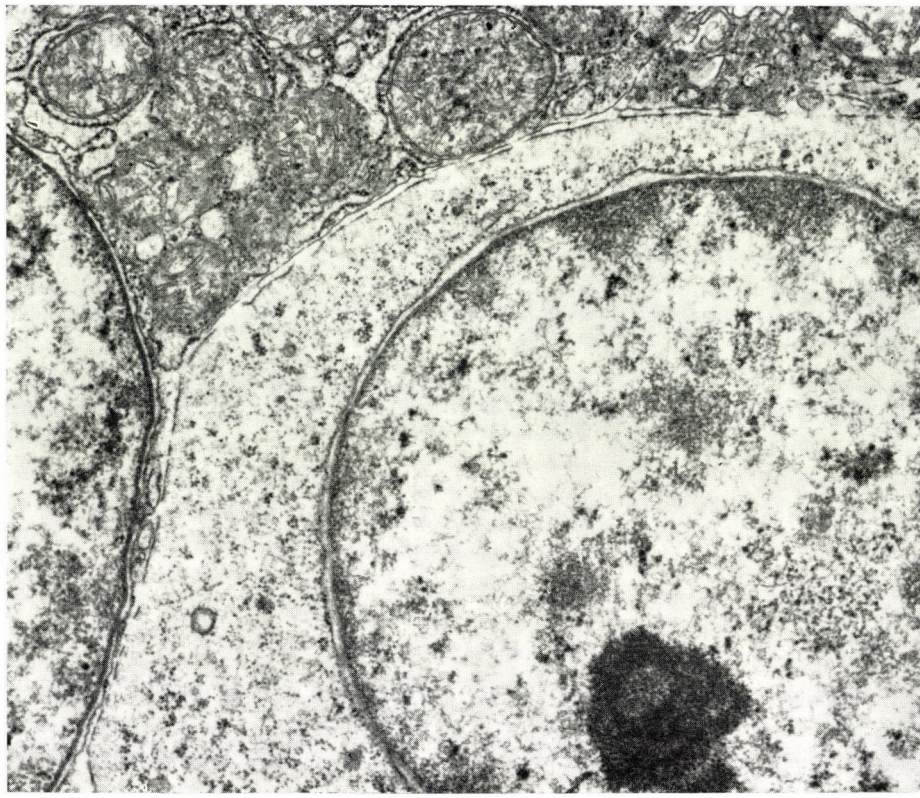

*Fig. 9.* Liver tumour cell with large, dark and electron-dense nucleolus ×19,800

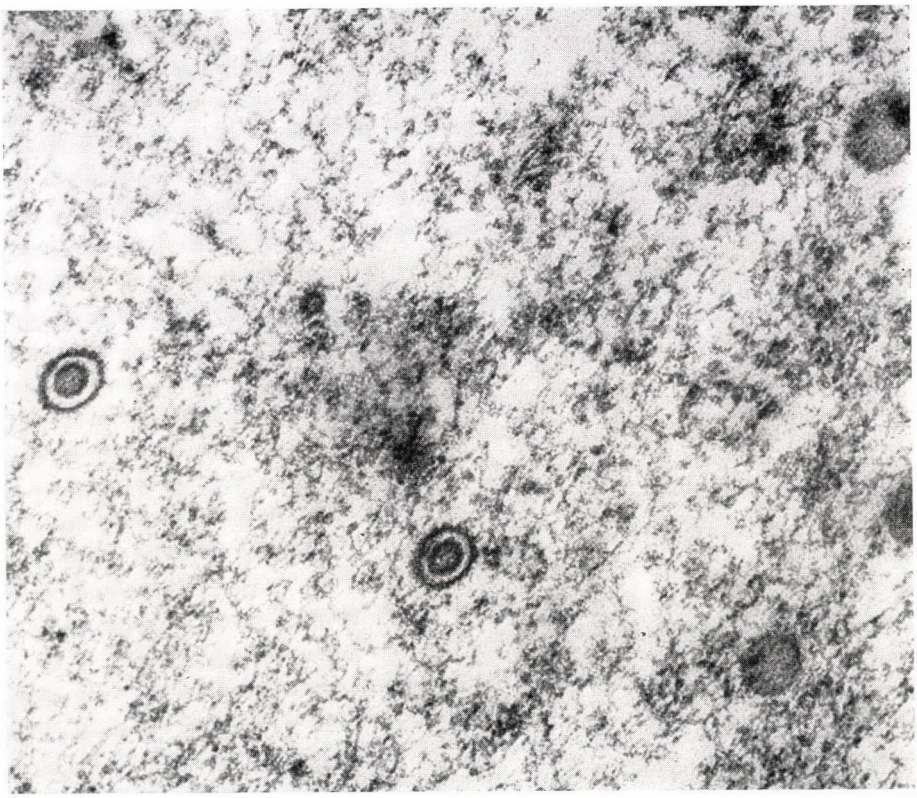

*Fig. 10.* Naked incomplete herpesvirus particles in the nucleus of an ovarian tumour cell ×80,400

## Summary

Early gross tumours and lesions produced in chicks after experimental infection with a virus isolated from acute Marek's disease (MD/7/1970) were examined with electron microscope. The cytomorphology of the tumour cells of lymphoid character are described. The tumour cells were poor in cytoplasmic cell organelles and often had characteristic nuclear invagination and evagination. They had a typical morphology characteristic of tumour cells.

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Address of the author: Dr. H. N. Thakur, Rajendra Agriculture University, Bihar Veterinary College, Patna 14, India

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# ELECTRON MICROSCOPE STUDY OF THE EARLY LESIONS IN MAREK'S DISEASE.

## II. HISTOGENESIS OF THE LESIONS

By

H. N. THAKUR

Department of Pathological Anatomy (Head: Prof. A. KARDEVÁN)
University of Veterinary Science, Budapest

(Received January, 21 1974)

The histogenesis of Marek's disease (MD) has been described by several authors (PAYNE and BIGGS, 1967; OKADA and FUJIMOTO, 1971; KARDEVÁN et al., 1973; SZÉKY and VÁNYI, 1973).

In the present paper we describe observations with electron microscope on the histogenesis of the lesions. The cytomorphology of the lymphoid cells, which form the main tumour lesions in acute MD, has been described in the accompanying paper (THAKUR, 1974). Furthermore, we have shown (KARDEVÁN et al., 1973) that the initial cell proliferation which leads to tumour formation in acute MD, starts from the walls of small blood vessels (undifferentiated mesenchymal tissues) in the visceral and peripheral nerve tissues. The present investigation at the ultrastructural level confirms our previous finding.

### Materials anf Methods

These were the same as described in the previous paper. Semi-thin sections were stained with toluidine blue.

### Results

In order to study the histogenesis of the MD lesions at the ultrastructural level, we observed especially the walls of small blood vessels, particularly the adventitial cells. On the basis of our previous results (KARDEVÁN et al., 1973), obtained with light microscopy, this site was considered to be the area of the initial proliferation after infection with the MD virus.

The results have confirmed the above observation. The cell proliferation was always in relation to, and seemed to start from the walls of the adjacent small blood vessels, irrespective of the organ in which the tumour was growing. The proliferated lymphoid cells were in connection with, and proliferated from the walls of the small blood vessels (Figs 2, 3, 4). The normal position and relation of the adventitial cell in liver are shown in Fig. 1.

The cell proliferation started initially from the perivascular areas (Figs 5, 6) and an infiltrative expansion of these cells finally led to the formation

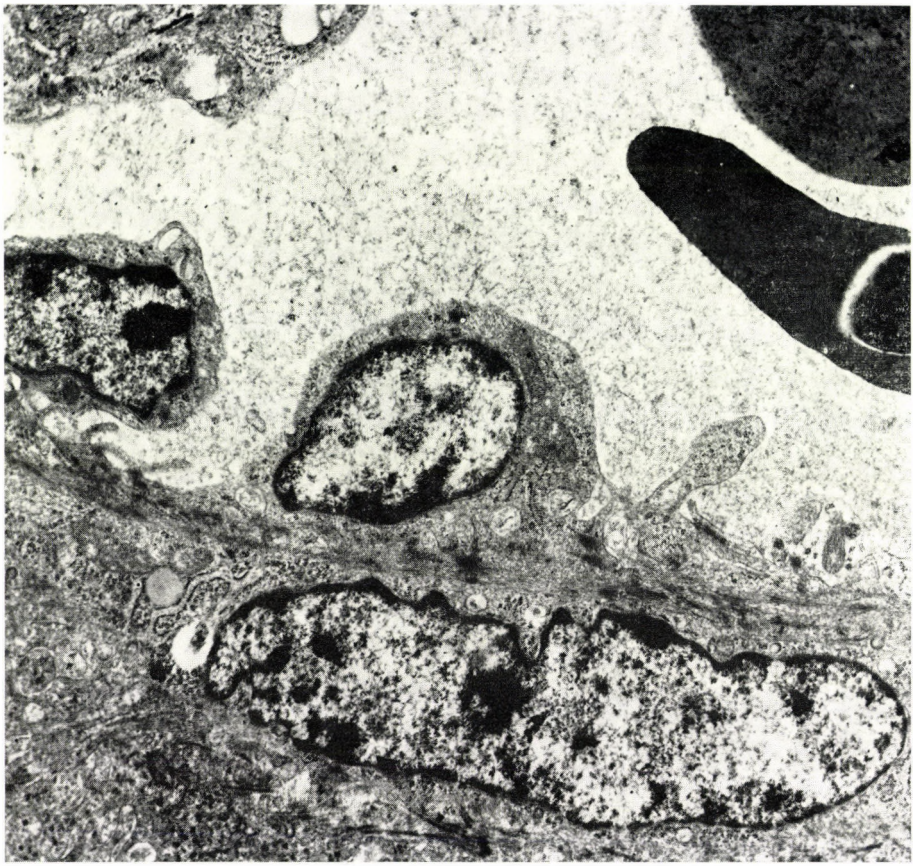

*Fig. 1.* Normal position of the adventitial cell in the perivascular space in liver ×9260

of tumour. The resulting tumours, especially those in the visceral organs, are visible to the naked eye.

In peripheral nerves two types of lesions were observed: one appeared to be thickened grossly and showed heavy infiltration of lymphoid cells microscopically, and another type that showed neither gross thickening nor any significant cell proliferation.

In the first type of the peripheral nerve lesions lymphoid cells were observed, similar to those seen in the visceral organs (Figs 7, 8, 9) and the cell proliferation, being perivascular in nature, was in good relation with the blood vessel walls (Fig. 9).

The second type of nerve lesion was characterized by vacuoles in the myelin sheath (Figs 11, 12). These probably indicate an injury to the outer

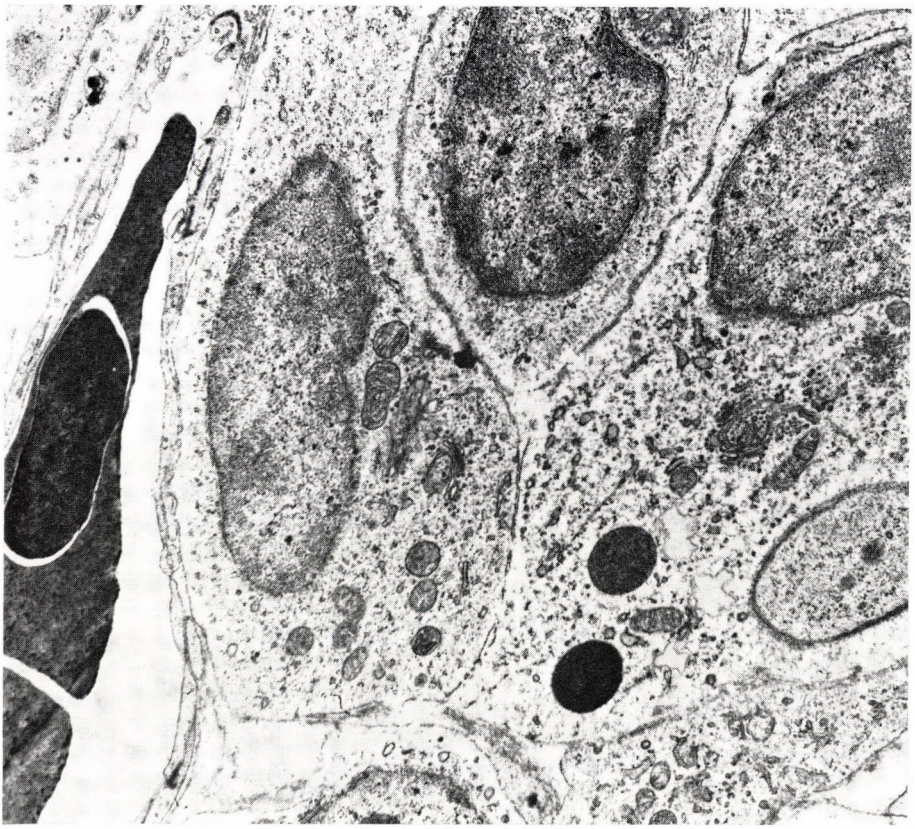

*Fig. 2.* Lymphoid cell proliferation in the perivascular area in a kidney lesion ×9260

membrane of the Schwann cells. This is observed well in comparison with the normal picture of the nerve in the control bird (Fig. 10).

## Discussion

The present investigation confirms our light microscopic findings concerning the histogenesis of the MD lesion (KARDEVÁN et al., 1973). It uniformly shows that the initial proliferation in MD is perivascular in nature. The perivascular cell infiltration starts from the walls of small blood vessels, as a proliferation of multipotent mesenchymal cells, viz. adventitial cells.

Lesions in the peripheral nerve in MD have been described in detail and classified into various types like A, B, and C (PAYNE and BIGGS, 1967), types I, II, III (WIGHT, 1969), $T_1$, $T_2$ and R types (OKADA and FUJIMOTO, 1971).

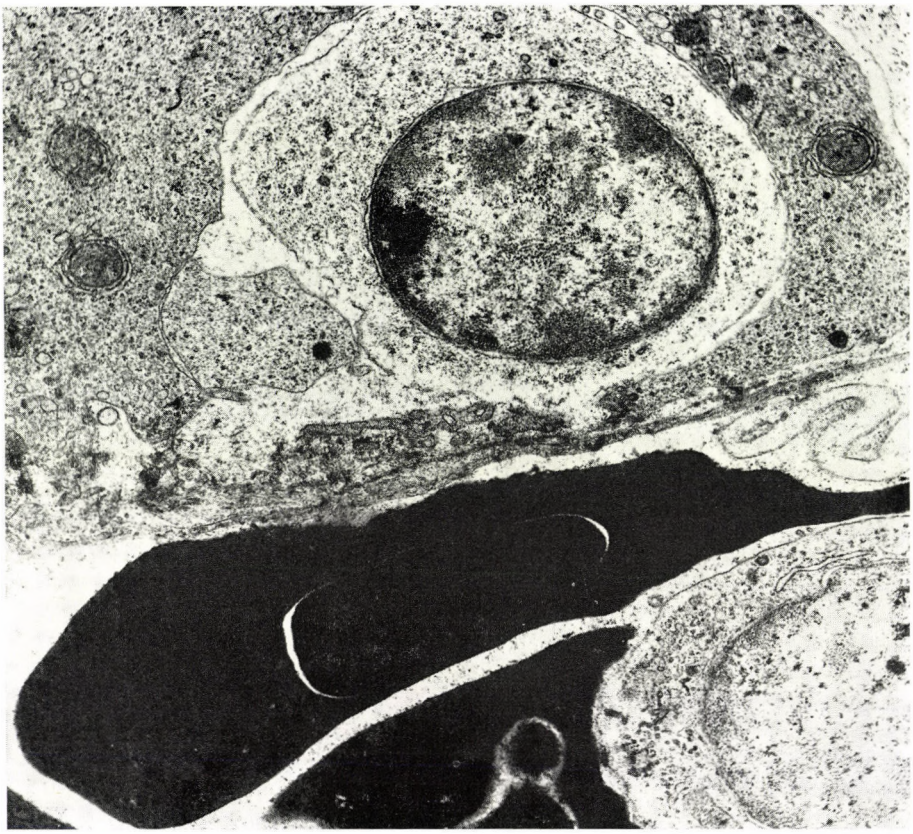

*Fig. 3.* Perivascular lymphoid cell proliferation in a tumour lesion of liver ×9260

These lesions were described and classified on the basis of the cellular proliferation and the lesions observed in various stages of the disease.

The $T_1$ type of peripheral lesion was characterized as proliferation of uniform small lymphoid cells, whereas the type $T_2$ lesion was described as consisting of pleomorphic cells, like small, medium and large lymphoid cell population with few reticulum cells. The type R lesions were described as infiltration by small lymphocytes, plasma cells, frequently accompanied by oedema and proliferation of Schwann cells (OKADA and FUJIMOTO, 1971).

The results of the present investigation on the histogenesis of the early lesions in acute MD indicate that the lesions are produced as a result of a chain reaction in which infiltration and proliferation of lymphoid cells are characteristic. Both types of cellular reactions, $T_1$ and $T_2$ types may be observed depending on the various stages of the disease. Thus the cellular reactions in

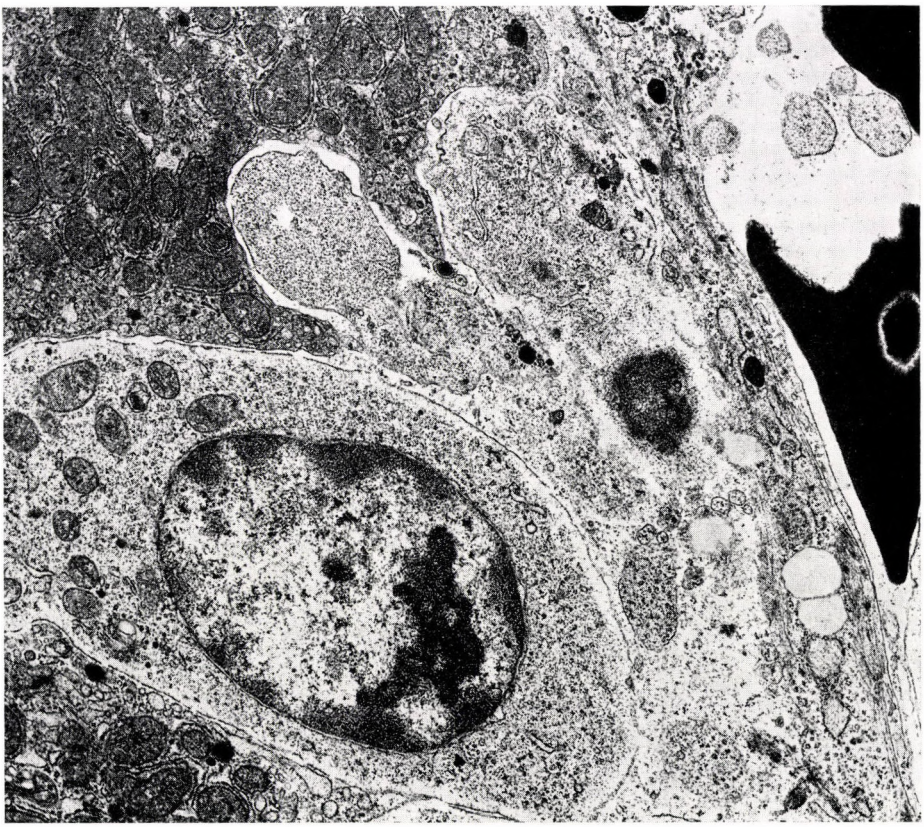

*Fig. 4.* Perivascular lymphoid cell proliferation with unipolar aggregation of mitochondria in liver × 9260

MD, both $T_1$ and $T_2$ types, either together or separately, can be observed, and this reaction is regarded here as characteristic of acute MD.

The R type lesions (OKADA and FUJIMOTO, 1971), on the other hand may represent a chain reaction, which is produced as a reactive inflammation in the peripheral nerve; it follows the initial reaction and appears after a severe regressive change in the nerve. This perhaps indicates an injury to the outer membrane of the Schwann cells, the myelin sheath (demyelination). However, in the early lesions of peripheral nerves, we failed to observe plasma cell infiltration.

This investigation further suggests that the histogenesis and nature of MD lesions in the visceral organs and peripheral nerves are initially the same. The initial cell proliferations were always observed in relation with, and starting from the connective tissues, situated in the walls of the small blood

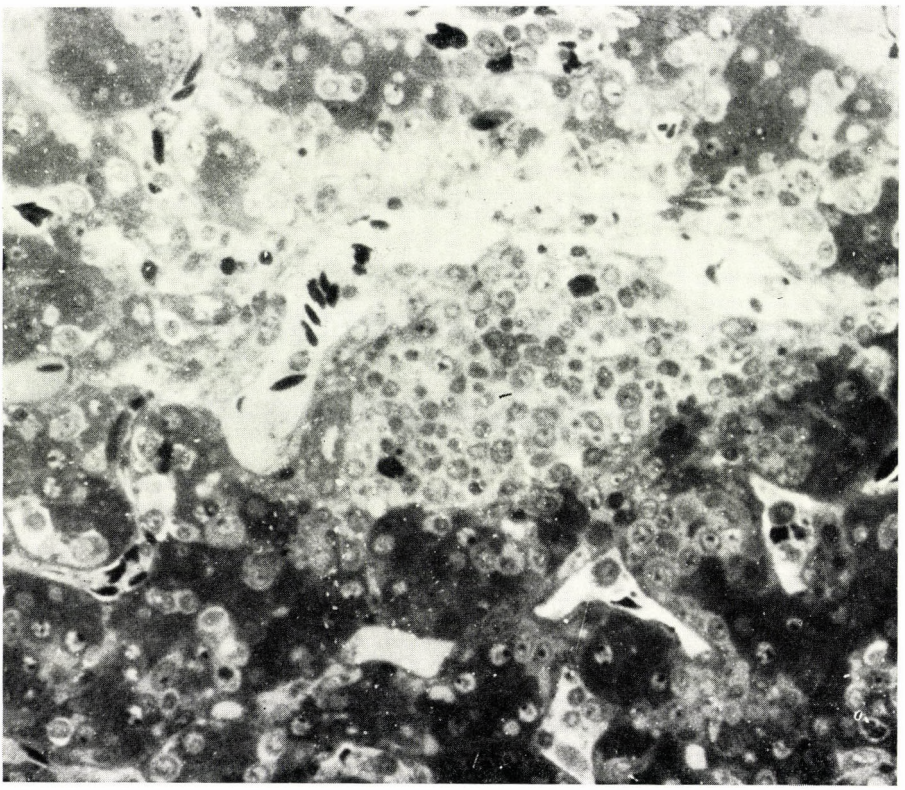

*Fig. 5.* Perivascular lymphoid cell proliferation in liver. Semi-thin section, toluidine blue stain ×900

vessels both in the visceral and peripheral nerve lesions. In the peripheral nerve also the initial proliferation was observed from the walls of the small blood vessels situated in the endoneural, epineural and perineural connective tissues. Sometimes only vacuolation was observed in the myelin sheath of nerves showing no marked lesions like cellular infiltrations of the lymphoid cells.

The other changes in the peripheral nerves in MD, like early activation of Schwann cells, lymphoblasts, and varied morphological changes in shape and size of the nucleus in tumour cells were in conformity with the earlier observations (WIGHT, 1969; OKADA and FUJIMOTO, 1971). However, virus particles were not observed in the lesions of the peripheral nerve.

*Acknowledgements.* The author is thankful to Prof. Dr. A. KARDEVÁN for his constant encouragement and help in carrying out this work. Thanks are also due to Mrs. Gy. PETHES, technician, for the electron micrographs and preparing the photographs.

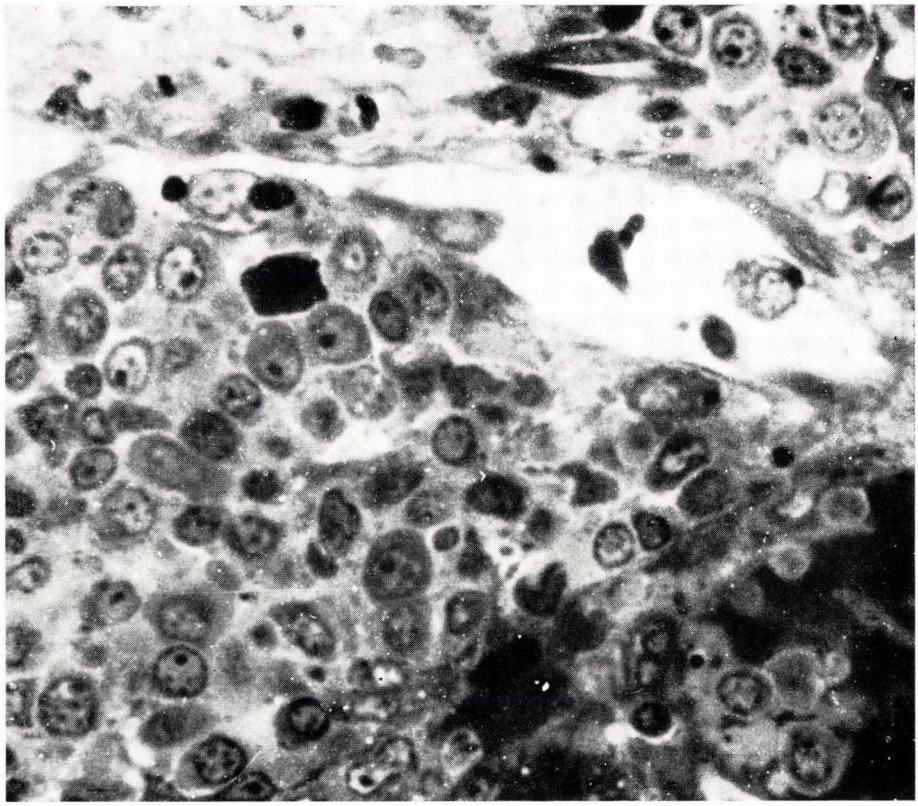

*Fig. 6.* Perivascular proliferation of pleomorphic, lymphoid cells in a liver lesion. Semi-thin section, toluidine blue stain ×900

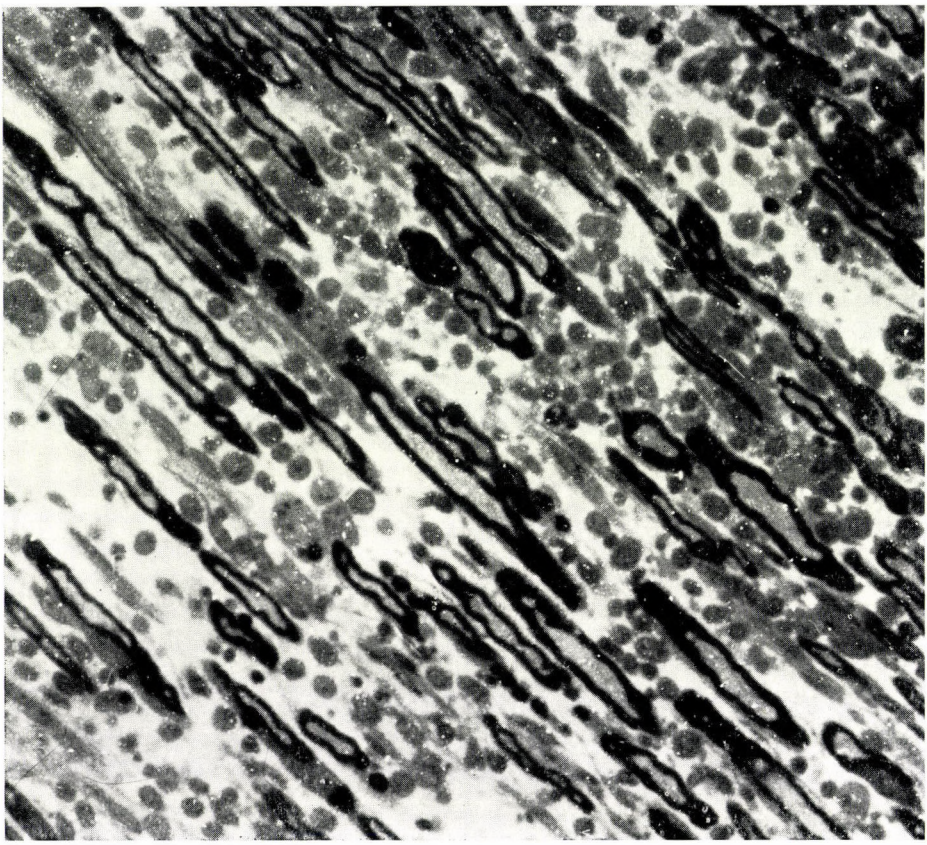

*Fig. 7.* Proliferation of lymphoid cells (pleomorphic) in a peripheral nerve lesion. Semi-thin section, toluidine blue stain ×900

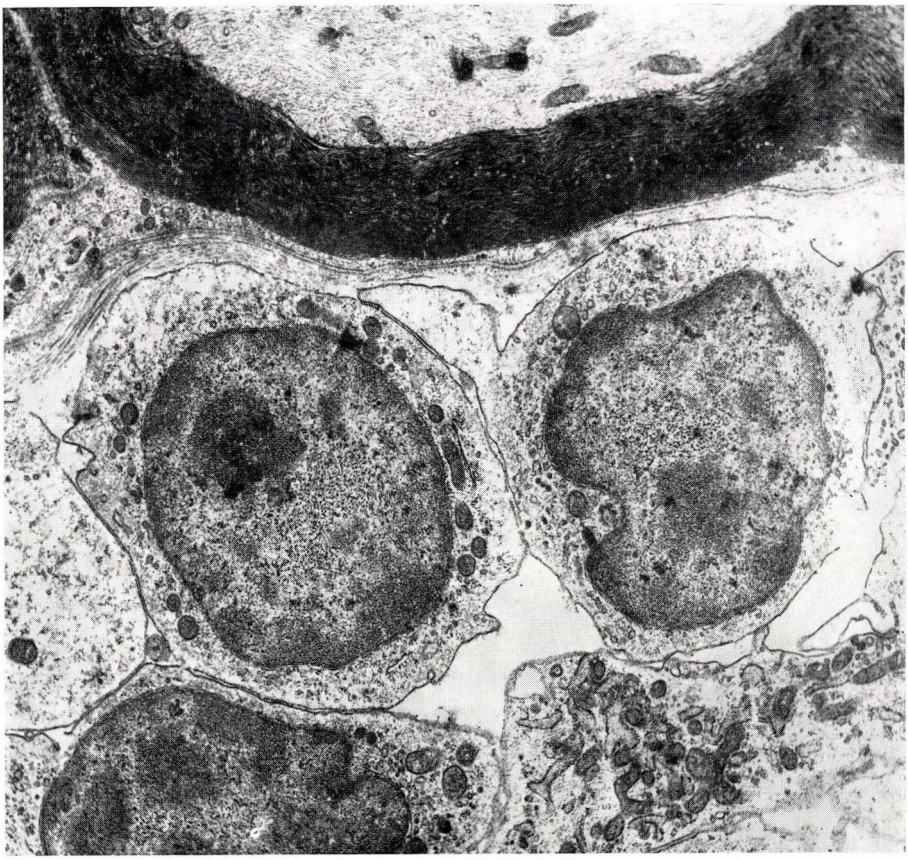

*Fig. 8.* Proliferation of lymphoid cells in a peripheral nerve lesion in MD ×9260

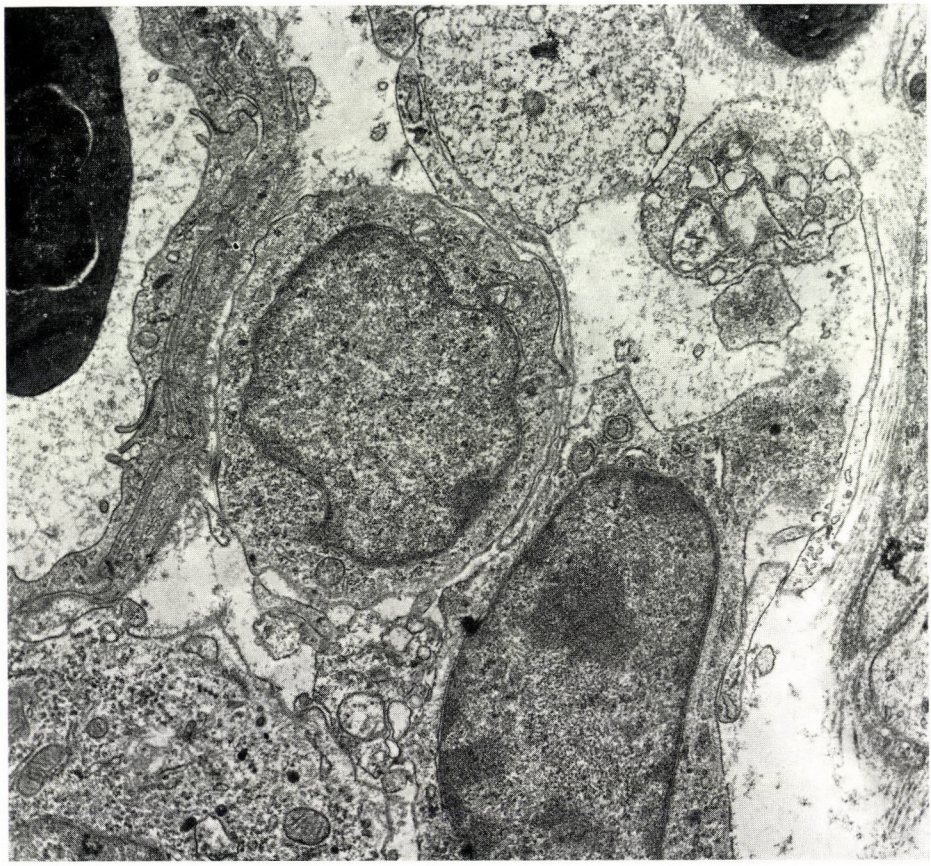

*Fig. 9.* Perivascular proliferation of lymphoid cells in a peripheral nerve in MD ×9260

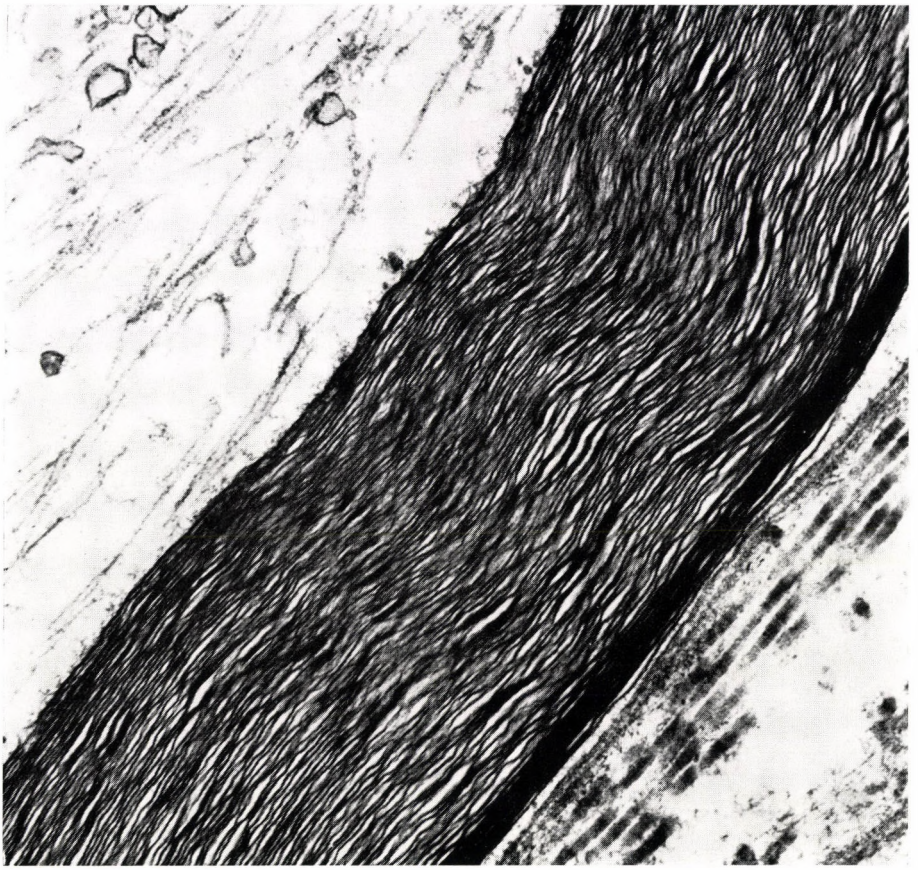

*Fig. 10.* Ultrastructure of a normal peripheral nerve in the control chick ×39,000

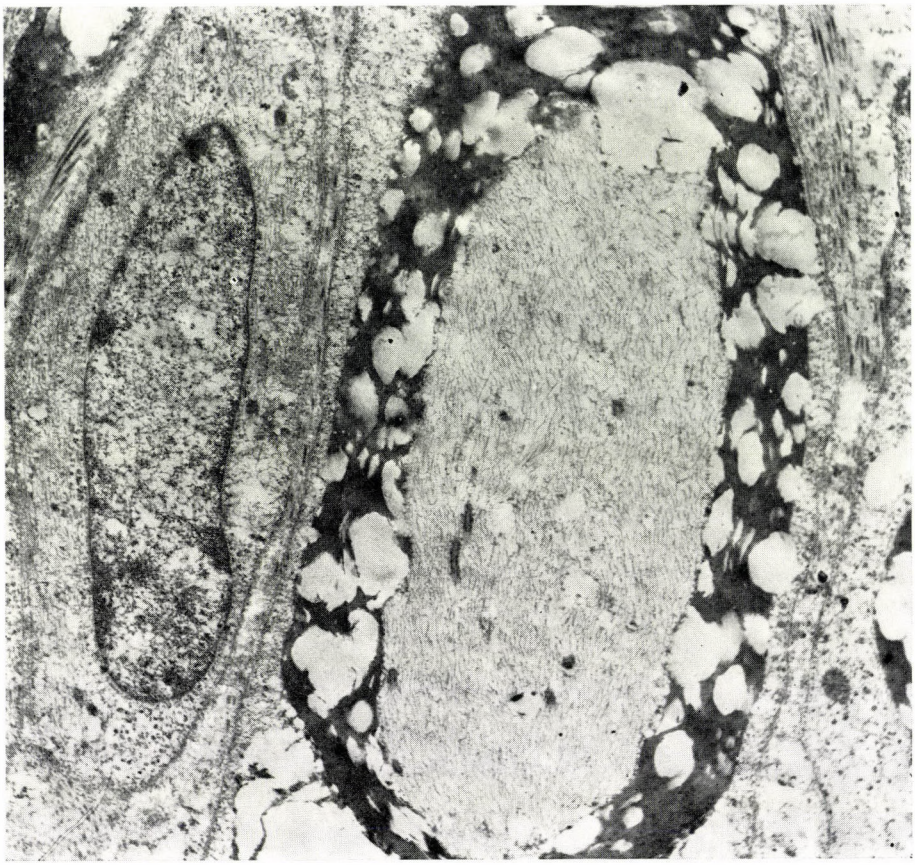

*Fig. 11.* Vacuoles in the myelin sheath of a peripheral nerve lesion in MD without cellular proliferation ×9260

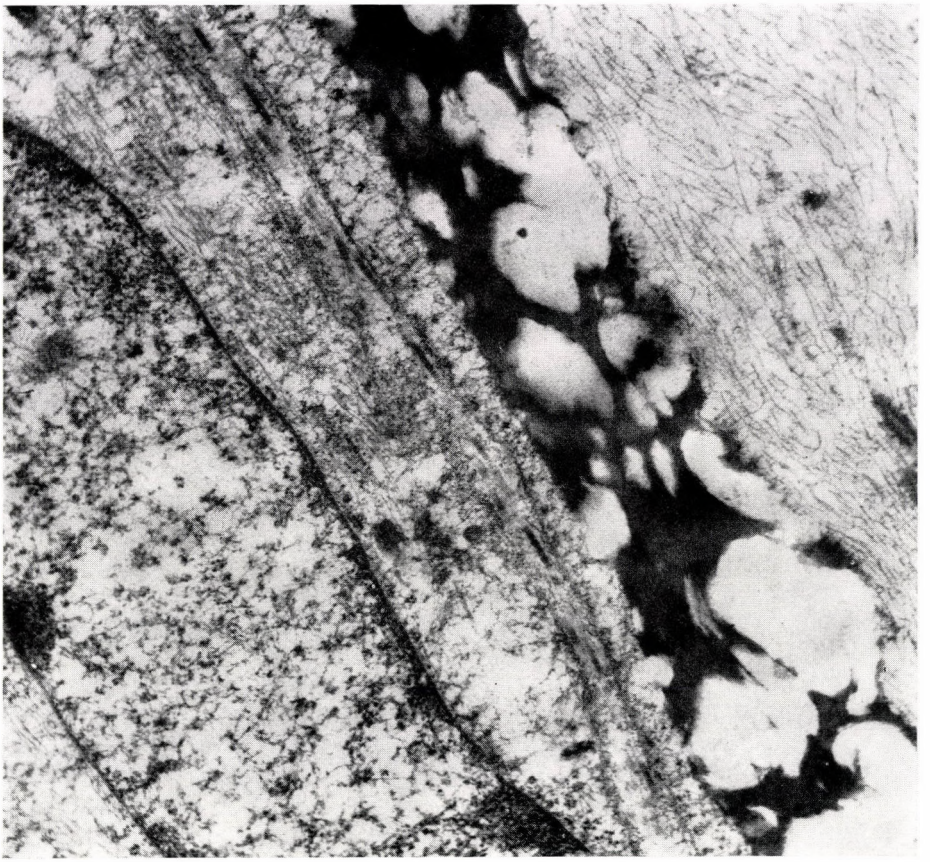

*Fig. 12.* Vacuolation in myelin sheath in a peripheral nerve lesion ×19,800

## Summary

An electron microscope study on the histogenesis of the early lesions in acute Marek's disease (MD) has confirmed our previous results obtained by light microscopy. The cell proliferations were perivascular in nature and were observed to start from the walls of small blood vessels, initially as a proliferation of multipotent mesenchymal cells, the adventitial cells. Infiltration by, and proliferation of pleomorphic lymphoid cells are characteristic features leading to tumour formation in MD. The cell proliferations in the visceral organs and those in the peripheral nerves were similar in nature.

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Address of the author: Dr. H. N. THAKUR, Rajendra Agriculture University, Bihar Veterinary College, Patna 14, India.

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# CHARACTERIZATION OF AN OVINE STRAIN OF PARAINFLUENZA-3 VIRUS ISOLATED FROM SHEEP SHOWING RESPIRATORY DISEASE

By

S. BELÁK and V. PÁLFI

Department of Epizootiology (Head: Prof. J. MÉSZÁROS), University of Veterinary Science and the Central Veterinary Institute (Head: A. VÁNYI), Budapest

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The first isolations of ovine parainfluenza-3 (PI-3) virus strains were reported by HORE (1966) in the U.K. and by DITCHFIELD (1966) in Canada. Isolations have been also made in Australia (St. GEORGE, 1969), in Bulgaria (HARALAMBIEV et al., 1969), in New Zealand (CARTER and HUNTER, 1970) and in the USA (PARKS et al., 1972).

This report is concerned with the characterization of a PI-3 virus isolated from Hungarian sheep showing respiratory disease.

## Material and Methods

Ten merino sheep, aged 4 months were transported to our Institute for experimental purpose. On the day of arrival nasal discharge, sneezing and accelerated respiration could be observed in the animals. The sheep showed these mild clinical symptoms for 8 days. The body temperature was by 0.5—1 °C higher during this period, than after ceasing of the symptoms. Nasal swabs were taken daily for 14 days. Sera were collected on the day of arrival and two weeks later.

The virus isolation was attempted from the nasal swabs using secondary ovine foetal kidney (OFK) cell cultures. The cells were grown in Eagle's Minimal Essential Medium (MEM, Difco) with lactalbumin hydrolysate (0.5%) and foetal calf serum (15%). Earle solution (Difco) was used as maintenance medium. Physicochemical properties and the type of viral nucleic acid were investigated by the method described before (BELÁK and PÁLFI, 1974). Plaque formation was observed in Petri dishes under agar or methylcellulose (Tylose, MH-4000, Hoechst) overlays in 5% $CO_2$ millieu at 37 C°. Identification of the isolate was attempted by the means of haemadsorption, haemagglutination (HA), haemagglutination inhibition (HI) and serum neutralization (SN) tests using specific antisera prepared in rabbits against the UMEA strain of PI-3 viruses and against the new isolate. Antisera were produced by intravenous inoculation of rabbits at weekly intervals for 4 weeks with 1 ml of virus sus-

pension ($10^{-6}$ $TCID_{50}$/ml) and bled two weeks after the final injection. Guinea pig erythrocytes were used in haemadsorption, HA and HI tests. Serological methods were carried out as described elsewhere (BELÁK and PÁLFI, 1974).

## Results

A viral agent was recovered from seven of the sheep. It could be isolated from the nasal swabs collected on the 1—6th days after arrival. Refractile and

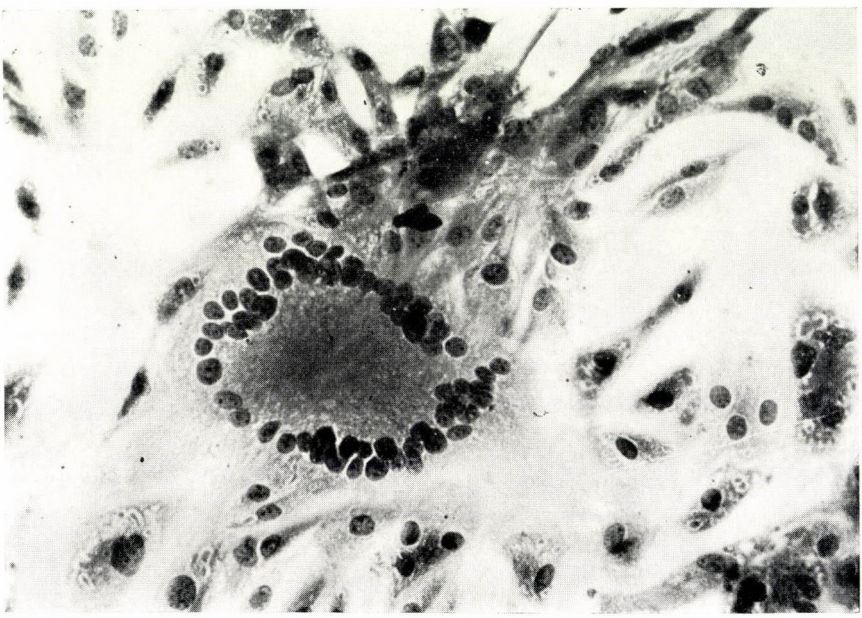

*Fig. 1.* Large syntitium and intracytoplasmic inclusion bodies. Ovine foetal kidney culture, 72 hours p.i. H.E. stain, 300 ×

elongated groups of cells were seen by native examination on the third day p.i. in the second passage of inoculated cell cultures. Eosinophylic intracytoplasmic inclusion bodies were found in haemalaun–eosin-stained preparations characteristic of PI-3 viruses (DEIBEL and HOTCHIN, 1961). Large syntitia formation was also observed (Fig. 1). In further passages syntitia were seen containing more than 40—50 nuclei. In physicochemical tests the isolate designated HOB/11 proved to be heat-labile at 56 C°, not stabilized in the presence of bivalent cations, sensitive to lipid solvents, pH 3, sodium deoxycholate and saponin (Table I).

**Table I**

Effect of physicochemical treatments on the infectivity titre of the HOB/11 virus

| Type of exposure | Infectivity titre ($log_{10}TCID_{50}ml$) | |
|---|---|---|
| | Pre | Post |
| 56°C 30 min | 7.0 | 1.25 |
| 50°C 60 min in distilled water | 7.0 | 4.50 |
| 50°C 60 min in M $MgCl_2$ | 7.0 | 1.75 |
| 20% Chloroform 16 hours 4°C | 7.0 | <1.0 |
| 0,125% Trypsin 30 min 37°C | 7.0 | 1.75 |
| Saponin | 7.0 | <1.0 |
| Sodium deoxycholate | 7.0 | <1.0 |
| pH 3 30 min 37°C | 7.0 | <1.0 |

The replication of the HOB/11 isolate and that of the known PI-3 virus (UMEA) was not inhibited in the presence of 50 $\mu$g/ml IUdR, while it was reduced in the case of the virus of Aujeszky's disease (KF/61 strain) (Table II).

**Table II**

Behaviour of HOB/11 isolate and known RNA and DNA viruses in the presence of IUdR

| Virus strains | Virus titre ($log_{10}TCID_{50}ml$) | |
|---|---|---|
| | IUdR | Control |
| HOB/11 | 7.0 | 7.0 |
| UMEA | 6.25 | 6.50 |
| KF/61 | <1.0 | 5.75 |

Plaques of an average diameter of one millimeter were observed under both overlays.

Haemadsorption tests were positive on monolayers inoculated with the HOB/11 strain. The virus fluid of these OFK cultures agglutinated the guinea pig erythrocytes at a titre of 1 : 64. HA titres were 1 : 16 when the virus was propagated in bovine kidney (BK) cultures. The HI titres were < 1 : 10 in the sera of the diseased animals on the day of arrival. In the convalescent sera 1 : 32—1 : 64 titres were found, one animal had a titre of 1 : 128.

A close relation was found between the HOB/11 isolate and the UMEA strain when reading the results of the HI and SN tests (Table III).

**Table III**

Result of HI and SN tests

| Viruses | Sera | | | |
|---|---|---|---|---|
| | HOB/11 | | UMEA | |
| | HI titre | SN titre | HI titre | SN titre |
| HOB/11 | 1024 | 256 | 512 | 512 |
| UMEA | 256 | 256 | 1024 | 512 |

## Discussion

The cytological changes observed in the infected cultures were comparable with those described for the PI-3 viruses. Not only intracytoplasmic inclusion bodies were observed, but also a very strong tendency in inoculated cultures for forming huge syntitia. In the physicochemical tests the HOB/11 strain proved to be sensitive to chloroform, indicating the presence of a lipoprotein envelope. Its further characteristics and the type of nucleic acid suggest that the HOB/11 isolate represents a paramyxovirus. In the serological tests it proved to be related to the UMEA strain of PI-3 viruses. The HOB/11 strain readily replicated not only in OFK cells, but also in BK cultures. It is noteworthy, that similar infectivity titres were found in both types of cell cultures, but the HA titres were always higher in the tissues of sheep origin. It seems to be necessary to study the further differences or similarities between the sheep isolate and PI-3 viruses of bovine origin.

The HOB/11 strain was isolated from sheep showing mild respiratory symptoms. On the basis of the significant rise of specific antibodies during the convalescent period it seems to be presumable that the isolated strain played an etiological role in indicating these symptoms. Around the date of transportation a mild respiratory disease occurred also in the farm from which the animals originated.

Considering that HORE and STEVENSON (1967, 1969) and ST. GEORGE and KIEFMAN (1972) already reported on the causative role of ovine PI-3 viruses in viral pneumonias of sheep, we also attach importance to investigate the pathogenicity of the new isolate in lambs, as well as to study the serological distribution of PI-3 viruses in the local sheep populations. This work seems to be necessary to be done because in a previous survey we have found that 52.8% out of 377 examined sheep sera had higher than 1 : 16 HI titres against the HOB/11 isolate.

FAYE and CHARTON (1967) reported pneumonic lesions in lambs infected with a bovine PI-3 virus. STEVENSON and HORE (1970) have found in their

experiments that calves were susceptible to an ovine PI-3 virus, and lambs also to a PI-3 virus of bovine origin. In the time of isolation of the HOB/11 strain from sheep, severe epidemics occurred in this country also among cattle populations most likely due to PI-3 viruses. According to these findings the possibility of the natural transmission between the two species cannot be excluded.

## Summary

A virus strain was isolated from the nasal swabs of 4 months old merino sheep showing a mild respiratory disease. The isolate designated HOB/11 was characterized in physicochemical and serological tests and it proved to belong to parainfluenza 3 viruses. According to the serological findings the isolate may be regarded as an etiological factor in respiratory disease of sheep. The possibility of transmission of PI-3 viruses between cattle and sheep populations should be brought up.

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Address of the authors: Dr. Sándor BELÁK, Dr. Vilmos PÁLFI 1143 Budapest, Hungária krt. 23, Hungary

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# VIRAL DISEASE OF GOSLINGS

## IV. CHARACTERIZATION OF THE CAUSAL AGENT IN TISSUE CULTURE SYSTEM

By

J. KISARY and D. DERZSY

Veterinary Medical Research Institute, Hungarian Academy of Sciences, Budapest
(Director: D. DERZSY)

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In the previous paper (DERZSY et al., 1970) a report was given on isolating a virus designated "B" from dead goslings in embryonated goose-eggs. The virus proved to be resistant to chloroform, heat and various chemicals. It failed to agglutinate the red blood cells of various animal species. Goslings infected in fields and experimentally became ill within 6—10 days with a high mortality. Muscovy ducks *(Cairina moschata)* and embryonated eggs were also susceptible to the virus. No other birds could be infected successfully. 9—11-day-old goose-embryos inoculated *via* the allantoic cavity were killed by the virus, as a rule, at the 3—8 days p.i. Meanwhile, attempts were made to adapt the virus to different kinds of monolayer tissue cultures with no success. Later on, it was stated that the virus replicated in goose-embryo fibroblast cells but no visible cytopathic effect was caused by it (HAHN, 1972).

This report describes the adaptation of the "B" virus strain to goose embryo fibroblast cell culture and its partial characterization, performed in this system.

### Materials and Methods

*Virus.* The 8th-egg-passaged virus of strain "B" was used in attempts for adaptation to fibroblast cell cultures. The allantoic fluid of the infected embryos served as inocula. The virus of 15—20th passage numbers in tissue culture was characterized physicochemically and electronoptically.

*Cell culture.* 14-day-old goose embryos were used for fibroblast cell cultures by standard methods. Virus culture medium was TCM-199 (Difco) supplemented with 0.5% lactalbumin hydrolyzate and 5% inactivated fetal calf serum. TC Earle solution (Difco) served as maintenance medium with 2% fetal calf sera. 100 mcg of streptomycin and 100 units of penicillin per ml were added to both media. Virus titrations were performed in usual manner and 0.2 ml was used as infection dose. 3 tubes per dilutions were inoculated before seeding of cells except for the determination of the nucleic acid type.

*Hemagglutination.* Human group 0, guinea pig, chicken, rat, mouse, goose and sheep red blood cells were attempted to agglutinate at 4, 22 and 37 °C.

The diluent used was PBS of pH 7.2—7.4. The incubation period lasted 4—6 hours.

*Electron microscopy.* 320 ml of tissue culture harvest were treated with fluorocarbon three times and centrifuged at 12,000 rpm for 60 min at 5 °C (Janetzki K24 centrifuge). The virus was pelleted by ultracentrifugation of 123,000 g for 90 min at 5 °C (Spinco model L2—65B, Type Ti60 rotor). The virus pellets were resuspended in 1 ml PBS of pH 7.2. The electron microscopic studies of the virus were made by Dr. KAPP at the Dept. of Pathological Anatomy of the Univ. of Veterinary Science, Budapest. Formvar-coated and carbon stabilized grids were covered with a small drop of the virus suspension. After a short drying period the grids were stained with a drop of 2% phosphotungstic acid (PTA) in distilled water adjusted to pH 7.0 by KOH. The grids were examined in a Tesla BS613 electron microscope.

*Chloroform sensitivity.* Virus suspensions were mixed with chloroform v/v and shaken for one hour at 37 °C. This was allowed to stand for 18 hours at 4 °C. Controls were set up with PBS instead of chloroform. The infectivity of both was determined in tissue cultures.

*pH-stability.* The pH-stability at pH 3 of the "B" virus strain was determined using a method described by PLUMMER (1963).

*Temperature sensitivity.* Glass ampoules containing the virus suspension were immersed into a waterbath of 56 °C for 30 min and 60 °C for ten minutes. At the end of the incubation period the ampoules were put into an ice bath and the virus was titrated parallel with the untreated control samples.

*The type and strandedness of the nucleic acid.* The type of the nucleic acid was determined by 5-iodo-2-deoxyuridine (IUDR) inhibition test. Tube cultures of not confluent monolayer were inoculated with 0.2 ml virus suspension and incubated for 3 hours at 37 °C. After that, they were washed twice with PBS and the medium, containing 50 mcg IUDR/ml was added. Infected control tubes were similarly prepared without IUDR. After 5 days the culture fluids were assayed for infectivity. Newcastle and pseudorabies viruses served as controls of known nucleic acid type.

The strandedness of the nucleic acid was tried to determine by acridine-orange staining according to the method described by MAYOR and HILL (1961).

## Results

*Virus propagation.* All attempts to adapt the egg-passaged virus to completely confluent monolayer tisssue cultures were without success. When inoculating the virus into the suspension of cells to be plated or monolayer of 70—80% confluency, a well-defined c.p.e. was seen both in native and stained preparations. After 72—96 hours p.i. shrunking and rounding up of the cells

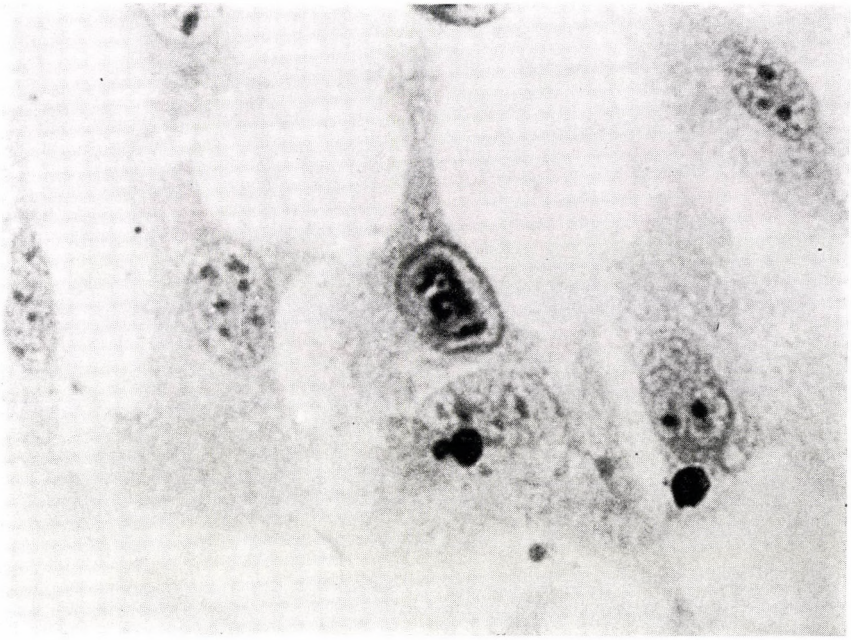

*Fig. 1.* Intranuclear inclusion body caused by "B" virus strain in goose-embryo fibroblast cells. Stained by hem.-eos. ×1000

were visible in different areas of the cell-sheet. Later on, the cytoplasm of the affected cells disappeared and the cells detached from the glass. In preparations stained by hematoxyline–eosin a large number of the nuclei contained an eosinophilic intranuclear inclusion body. This was first detected between 36—48 hours p.i. (Fig. 1).

*Hemagglutination.* The virus failed to agglutinate any of the erythrocytes tested at different temperatures.

*Heat resistance.* No decrease in the infectivity of the virus could be detected after exposing it to 56 °C for 30 and 60 °C for 10 min (Table I).

*Chloroform sensitivity.* The infectivity of the virus was not decreased by chloroform treatment, moreover, a slight increase could be achieved in the titer of the virus (Table I).

*pH-stability.* No change in the virus infectivity was detected after exposing the virus to pH 3 for 60 minutes at 37 °C (Table I).

*Electron microscopy.* Small particles of 20—22 nm in diameter were seen electron microscopically using negative contrast staining technique. The viral particles were aggregated and they were empty in a high percentage. No envelope could be seen and the particles were hexagonal in shape (Fig. 2). These findings were supported by those obtained at the IFFA Institute, France,

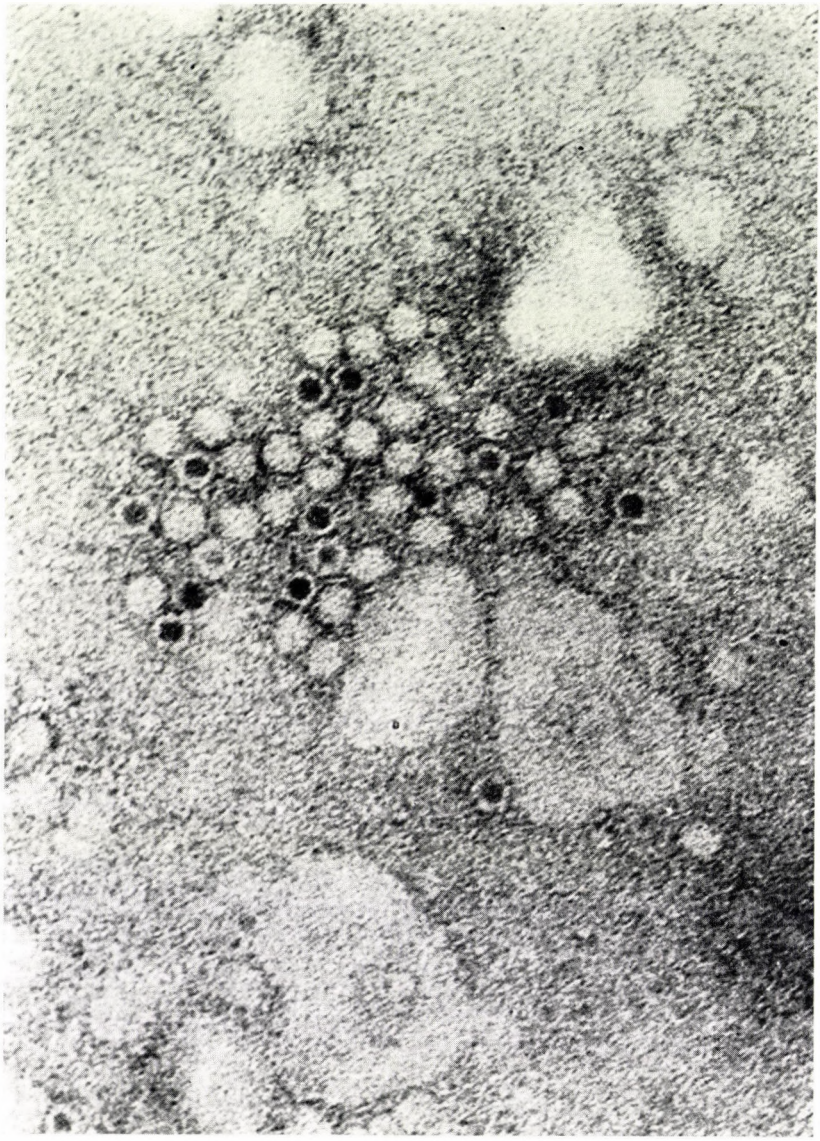

*Fig. 2.* Electron micrograph of "B" virus strain by negative contrast technique. ×238200

**Table I**

Results of the physicochemical tests

| Tests | Infective titers+ | |
|---|---|---|
| | treated | control |
| Heat | | |
| 56 °C for 30 min | $10^{4.5}$ | $10^{4.5}$ |
| 60 °C for 10 min | $10^{4.39}$ | $10^{4.5}$ |
| Chloroform | $10^{5.5}$ | $10^{4.75}$ |
| pH 3 | $10^{4.5}$ | $10^{4.5}$ |
| IUDR | $10^{0.39}$ | $10^{3.5}$ |

* The titers were calculated by method of REED-MUENCH (1938)

by Dr. TEKTOFF who found viral particles only in the nuclei in the ultra-thin-sectioned preparations (personal communication).

*Nucleic acid type.* A significant decrease was found in the titer of the virus replicated under media containing 50 mcg/ml IUDR. This indicates a DNA nature of the nucleic acid (Table I). The strandedness of the nucleic acid of the "B" virus strain could not be specified by the method used since the results obtained were unconclusive.

## Discussion

The "B" virus strain has been isolated in 9—10-day-old goose embryos inoculated via the allantoic cavity. When older embryos were used only a slight virus replication could be achieved. Meanwhile, the attempts to adapt the virus to goose embryo-kidney and fibroblast monolayer tissue cultures were with no success. At the same time, when the virus was inoculated into suspension of cells to be plated or not yet confluent monolayer, the virus replicated in cells with visible and well-defined c.p.e. This finding is similar to that described by PARKER et al. (1970) on the MVM (minute virus of mice) a member of the parvovirus group. It was also reported by BACHMANN (1972) and SIEGL and GAUTSCHI (1973) that other parvoviruses also needed dividing cells for the replication.

The cytological studies revealed that the "B" virus strain replicated in the nuclei of the infected cells causing intranuclear inclusion bodies of Cowdry A type. The morphology of the viral particles showed hexagonal appearance and it measured 20—22 nm in diameter. No envelope was demonstrated electron microscopically. The virus proved to be resistant to the chloroform and heat

treatment. The infective titer of the virus was not influenced by the pH 3. The "B" virus strain contained DNA since the replication could be prevented by adding IUDR to the culture media.

SCHETTLER (1971) reported on isolating a small DNA-containing virus from goslings being affected by similar disease. However, SCHETTLER (1973) described that his isolate caused intranuclear inclusion bodies in the affected cells and "typical formation of syntitia" in the same culture. We do not regard the latter as specific to the c.p.e. caused by the goose parvovirus, since, it can be often observed in uninfected cultures, especially, when incubated for a long period. On the other hand, it has not been excluded by SCHETTLER (1973) that his isolate involves two different viruses. This seems to be backed by his electronoptical findings, that is, a particle of 40—45 nm and another with a diameter of 20—22 nm were seen.

DANNACHER et al. (1974) recently reported on characterization of a small DNA-containing virus isolated from goslings. It is worth to be noted, that no intranuclear inclusion bodies were observed by him in the infected cultures, but pycnosis of the affected cells was described as characteristic type of c.p.e.

On the basis of all properties investigated and described above we propose that the "B" virus strain should be regarded as a possible member of the parvovirus group.

*Acknowledgements.* The authors wish to thank Assoc. Prof. Dr. P. KAPP and Mrs. Éva PETHES for the electronmicrograph. The excellent technical assistance of Miss Magdolna SZAMOSI and Mrs. Katalin VÖRÖS is also acknowledged.

## Summary

The virus strain "B" isolated from dead goslings has been adapted to goose embryo fibroblast cell culture. The virus caused eosinophilic intranuclear inclusion bodies in the affected cells. In the physicochemical tests it proved to be resistant to the chloroform and heat treatment and stable at pH 3. The type of the nucleic acid was DNA as it was determined by IUDR inhibition test. The virions measured 20—22 nm in diameter in negatively stained preparation, electron microscopically. Based on these properties we propose that the "B" virus strain should be regarded as a possible member of avian origin of the parvovirus group.

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Address of the authors: Dr. János KISARY, Dr. Domokos DERZSY, 1143 Budapest, Hungária krt. 23, Hungary

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# STUDIES ON SOURCE OF *TOXOPLASMA* INFECTION IN CAPTIVE KANGAROOS

By

M. DOBOS-KOVÁCS, J. MÉSZÁROS, L. PELLÉRDY and A. BALSAI

Department of Pathology (Head: Prof. A. KARDEVÁN), Department of General Zoology and Parasitology (Head: Prof. T. KOBULEJ) and Animal Hospital (Head: L. FÁBIÁN) of the Budapest Zoo and Botanical Gardens, Budapest

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*Toxoplasma* infection is known to occur in various species of kangaroos kept in zoos: it was observed in *Macropus bennetti* (RATCLIFFE and WORTH, 1951; GRÜNBERG, 1959; MÖLLER, 1962; MANDELLI et al., 1966), *Macropus rufus* (HACKEL et al., 1953), *Macropus rufogriseus* (RATCLIFFE, 1953; HILGENFELD, 1966), *Macropus robustus* (RATCLIFFE, 1953), *Protemnodon bicolor* (THOMPSON and REED, 1957), *Megaleia rufa*, *Macropus giganteus* (HILGENFELD, 1966) and in a species not nearer identified by BIERING-SÖRENSEN (1953).

Infection was sometimes suspected while the diseased animal was alive (JAKSCH, 1959), but as a rule it was not recognized until post mortem or histological examination (RATCLIFFE and WORTH, 1951; GRÜNBERG, 1959; MÖLLER, 1962; MANDELLI et al., 1966, etc.).

Evidence was also presented by successful transmission of *Toxoplasm* to albino mice, by administration of organ suspension from dead kangaroos into the abdominal cavity (MÖLLER, 1962) and/or brain (MANDELLI et al. 1966) of the experimental animals. The preliminary diagnosis could be confirmed on the basis of the appearance of developmental stages in the abdominal cavity and — more often — of cysts in the brain.

The source of the *Toxoplasma* infection of captive kangaroos could not be identified in the previous cases, although certain authors (THOMPSON and REED, 1957; HILGENFELD, 1964) offered a hypothetical explanation, but failed to confirm it.

In the present case, *Toxoplasma* infection was demonstrated in three dead kangaroos by histopathological examination of certain organs and experimental transmission to a cat, and the possible source of infection could also be traced.

## Experimental

### *Materials and methods*

*Post mortem* examination of the carcases of three derby kangaroos (*Thylogale (= Wallabia) eugenii*) was performed in the Department of Pathology.

Specimens of spleen, liver, kidneys, adrenals, pancreas, stomach, various intestinal segments, mesenteric lymph nodes, urinary bladder, lungs, heart muscle, brain, spinal chord, diaphragmal muscle and several skeletal muscles were secured for histological examination. The organs were fixed in 10% formalin, embedded in paraffin and the sections were stained with haematoxylin and eosin.

*Toxoplasma* cysts were isolated by feeding pieces of certain organs (brain, spinal chord, heart and diaphragmal muscle) of the carcases to a toxoplasma-free young cat. *Toxoplasma gondii* oocysts were collected from the stool of the cat by flotation and were administered orally to albino mice. Brains of mice killed at various periods after the 14th post-infection day were homogenated and *Toxoplasma* cysts were demonstrated microscopically in unstained smears prepared from brain suspensions. A similar procedure was employed in investigations into the source of infection.

*Clinical observations*

Three derby kangaroos, one male and two females, kept in the same run in the Budapest Zoo, developed nervous symptoms (disturbance and incoordination of movements) almost simultaneously in September 1973. The animals also showed listlessness and loss of appetite and one even had dyspnoea. One kangaroo died on the second day after the manifestation of the symptoms, the other two succumbed on the tenth day. The latter two animals showed progression of the movement disturbance and gradual loss of condition

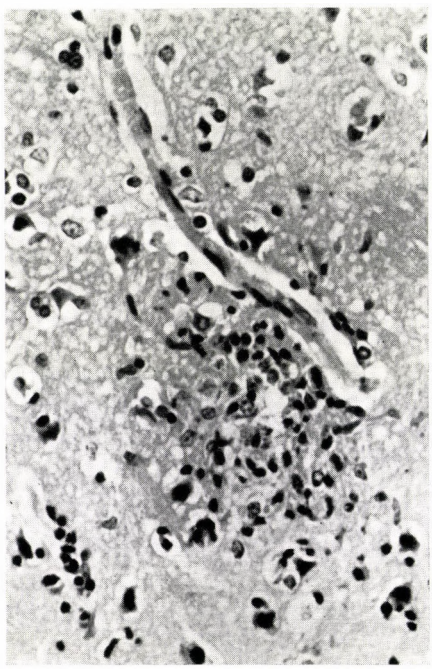

*Fig. 1.* Loose focal lesion, consisting of glia cells, nearby a cerebral vessel. HE stain, about ×275

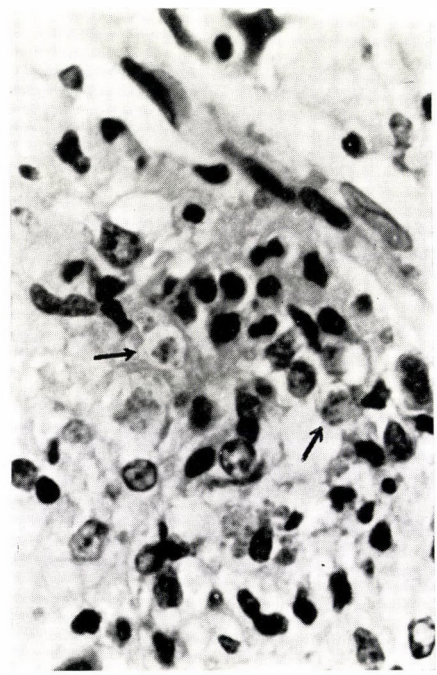

*Fig. 2.* Magnified detail of Fig. 1. Injured cells localizing inside the glia cell focus harbour single tachyzoites and groups of tachyzoites surrounded by a parasitophorous vacuole (arrow). HE stain, about ×700

until death. During the last days, there was a flaccid paralysis of certain muscle groups and compulsive movements appeared in the terminal stage. Exclusively symptomatic treatment was applied.

*Post-mortem and histopathological examinations*

No notable gross lesions were found on *post-mortem* examination.

On microscopic examination of brain and spinal chord from the animal which had died first, circumscribed, loose foci, consisting chiefly of glia cells, were found to occur sporadically in various areas, above all along the vessels (Fig. 1). Inside the foci the glia cells and infrequently also nerve cells showed regressive phenomena (nuclear pycnosis and lysis, rarefication of cytoplasm and necrosis of the cell). Solitary tachyzoites and groups of tachyzoites established themselves intracellularly, the latter being usually surrounded by a parasitophorous vacuole (Fig. 2). Only few *Toxoplasma* cysts were found in the brain and spinal chord. The two kangaroos, which had died later, also showed the sporadic focal proliferation of glia cells in brain and spinal chord. The localization of the foci was again not characteristic, but they were more

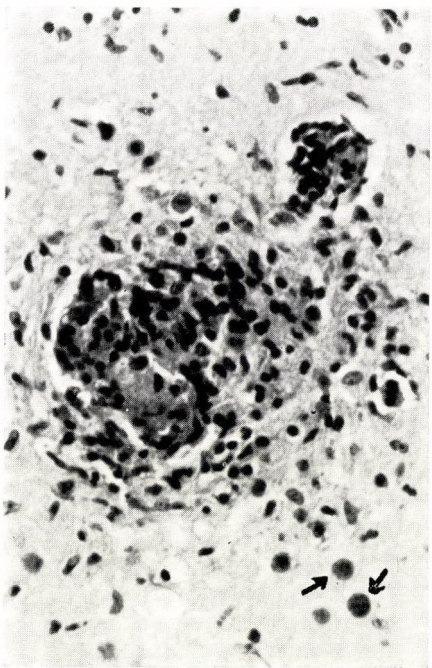

*Fig. 3.* Circumscribed glia cell proliferation in the brain. Note necrotic cells in centre of focal lesion and the *Toxoplasma* cysts localizing nearby (arrow). HE stain, about ×275

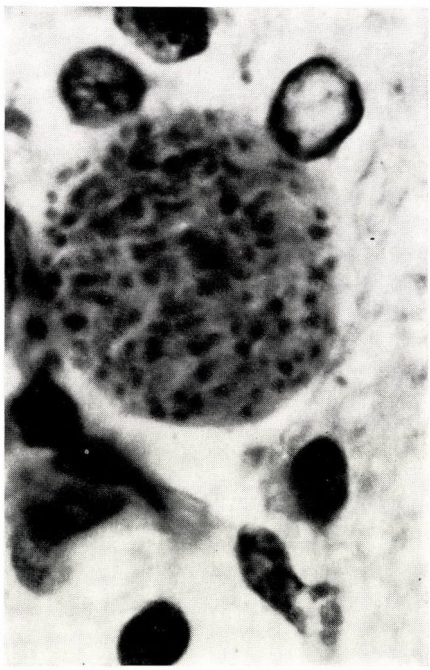

*Fig. 4. Toxoplasma* cyst localizing nearby a cerebral vessel. HE stain, magnification about ×1800

numerous than after the shorter course and the densely arranged glia cells surrounded the necrotic brain areas (Fig. 3). *Toxoplasma* cysts did not usually occur inside the foci, but outside the latter chiefly free cysts of various sizes were frequently found (Fig. 4). There was also a perivascular histio-lymphocytic infiltration in certain non-characteristic localizations.

The heart muscles of the three animals showed different degrees of change. The circumscribed focal lesions and often also the adjacent interstitial



*Fig. 5.* Circumscribed inflammatory cell reaction in the heart muscle. Low magnification view, about ×40

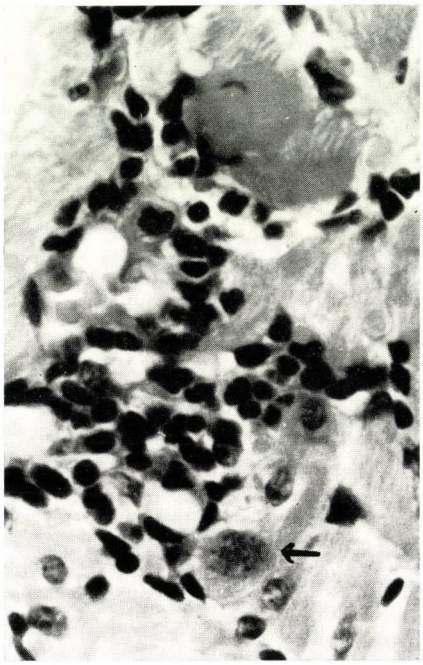

*Fig. 6.* A group of tachyzoites localizing in a heart muscle cell inside the area of the inflammatory cell reaction (arrow). HE stain, about ×700

tissue were infiltrated by histiocytes and lymphocytes (Fig. 5). In areas of acute or semi-acute myocarditis or nearby, large groups of tachyzoites localized in muscle cells showing regressive lesions (Fig. 6). Varying numbers of tachyzoite groups established themselves in myocardial cells farther away from the lesion.

Similar focal inflammatory lesions and intracellularly localizing groups of tachyzoites were also found in the tongue muscle.

One or both adrenals showed circumscribed necrotic lesions, involving chiefly the cortical part (Fig. 7). Nuclear pycnosis, rhexis and lysis and rare-

*Toxoplasma* cysts occurred in various segments of the small intestine, localizing in the propria of the mucosa, which appeared intact and did not show inflammatory reaction around the cysts.

Bodies reminding of *Sarcosporidium* cysts were found in the skeletal muscles, diaphragmal muscles and in oesophageal muscles. The parasites had an elongated shape and some showed indications of a compartmental inner fication of the cytoplasm took place in those cells which harboured single tachyzoites or groups, and free *Toxoplasma* cysts were localizing between

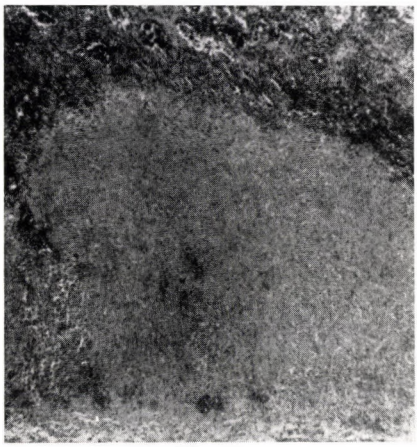

*Fig. 7.* Necrotic focal lesion in adrenal cortex. Low magnification view, about ×40

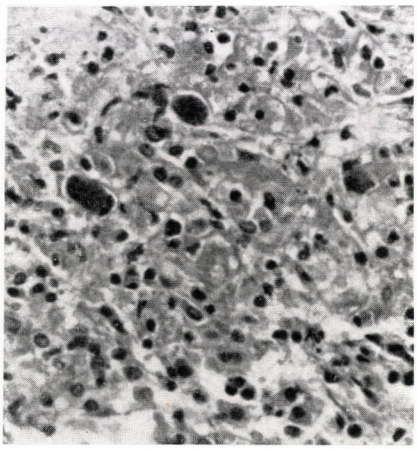

*Fig. 8. Toxoplasma* cysts in necrotic field of adrenal. HE stain, about ×275

the deteriorating cells (Fig. 8). The normal appearing tissue in the surroundings of fresh necrotic foci showed only hyperaemia.

Circumscribed necrotic foci, containing nuclear debris, were also found in the mesenteric lymph nodes. No developmental stages of *Toxoplasma* could be detected either in the foci or in the adjacent intact tissue.
structure. No inflammatory cellular reaction was seen around those muscle cells which harboured the *Sarcosporidium*-like cysts. Such bodies were also found in single muscle cells of myocardium and tongue.

No microscopic lesions were found in organs other than the above.

### *Isolation of* Toxoplasma *from the carcases of the kangaroos*

Specimens of brain, spinal chord, myocardium and diaphragmal muscle of the two kangaroos which died after illness for ten days, were fed to a young cat, which was free from *Toxoplasma* to judge from preceding faecal examinations. Throughout the experiment and for two weeks before it, the

cat was kept in isolation and was fed with great care to avoid intercurrent *Toxoplasma* infection. Seven days after the ingestion of organ pieces from the carcases, the cat began to pass *T. gondii* oocysts with the faeces and continued to discharge them for several subsequent days.

Mice could be infected experimentally by oral administration of oocysts collected from the cat. *Toxoplasma* cysts appeared in the brain of the mice at about 14 days after infection. The strain has been maintained in mice ever since.

*Investigations into the possible source(s) of infection*

A few days after the death of the kangaroos, a local investigation was made in the kangaroo premises of the Zoo. In the fodder store, which consisted of several rooms, including a preparatory room, a she-cat with two 6-week old kittens was found, which lived there permanently. Dry cat faeces was found in all rooms and samples of it contained a medium amount of *T. gondii* oocysts. A few oocysts were also found in the freshly passed faeces of the mother cat. The latter ceased to discharge *T. gondii* oocysts after kept in isolation for a few days. Faecal examination had negative results with the two kittens.

Mice were infected orally with *T. gondii* oocysts obtained from the dry cat faeces collected at the spot. Four days after infection the mice died in a severe pulmonary haemorrhage, because apart from *T. gondii* oocysts many embryonated (infective) *Toxocara mystax* eggs were present in the infectious material. In the mouse, being a paratenic host of the latter parasite, the larvae released from the eggs, penetrated across the intestinal wall, and caused severe lung injuries in the course of migration. Homogenates prepared from mesenteric lymph nodes and liver of the dead mice were administered orally or intraabdominally to further mice. Twenty-one days later *Toxoplasma* cysts were found in unstained smears of brain suspensions from both groups of mice.

Mice infected orally with *T. gondii* oocysts isolated from fresh cat faeces had *Toxoplasma* cysts in the brain two weeks after infection.

Twenty common mice, captured in the fodder store, were killed and their brains were examined for the presence of *Toxoplasma* cysts, but none were found. Blind transfers of brain homogenates from these animals were not made.

Unfortunately, the fodder supply from which the kangaroos had been fed before their illness run out completely by the time of the examinations.

## Discussion

All three kangaroos were found to have died in acute or semi-acute, generalized *Toxoplasma* infection.

Unlike most cases reported by other authors, the carcases did not show necrotic-inflammatory change or ulceration in the digestive tract and generally no gross lesion was found in any organ. Microscopic lesions related to the presence of *Toxoplasma* occurred above all in the brain, spinal chord, heart and tongue muscles, adrenals and mesenteric lymph nodes. The most frequent changes were a proliferative, acute or semi-acute, focal inflammation (in brain, spinal chord, heart and tongue muscle) and circumscribed focal necroses (in adrenals and mesenteric lymph nodes). Developmental stages of *Toxoplasma* (intracellularly localizing solitary tachyzoites or groups of tachyzoites, less often cysts) were found inside or nearby the lesions, occasionally also in distant organs (e.g. in the propria of the small intestine). The microscopic lesions were in every respect similar to those described in other reports of *Toxoplasma* infections in kangaroos. In all three cases, the brain and spinal chord were the most affected parts; this accords well with the clinical observations.

However, apart from developmental stages of *Toxoplasma*, sac-like bodies reminding of *Sarcosporidium* were also present in the heart and tongue muscle, oesophageal muscle layer, diaphragmal muscle, and certain cell groups of the skeletal muscles.

Isolation of *Toxoplasma* from the carcases is generally made by inoculation of organ homogenates into the abdominal cavity of mice. In this instance, isolation was attempted by oral administration of the infectious material to a susceptible young cat. The *T. gondii* oocysts passed by the cat were transmitted orally to mice. These successful transinfection experiments accord well with the recent knowledge about life cycle of the *Toxoplasma*. It appears that the probable concurrent infection with *Sarcosporidium* did not interfere with the results of the infection experiments.

Examinations on the spot showed that the most probable source of infection had been the she-cat which had lived with her two kittens in the fodder store from which the kangaroos had been supplied. *T.gondii* oocysts were demonstrated in dry cat faeces found all over the store and in the fresh stool of the mother cat as well. The low number of oocysts in the faeces and the fact that the cat ceased to discharge oocysts a few days after being examined regularly indicate that it had older *Toxoplasma* infection.

The simultaneous severe infection of the three kangaroos may well have resulted from the consumption of fodder (probably the grits) heavily contaminated with oocyst-containing cat faeces.

Apart from the known susceptibility of kangaroos to *Toxoplasma* infection, the disease and death of all three animals kept in the same run may chiefly have been due to a massive infestation.

The illness of the kangaroos was neither preceded nor followed by similar infection of other animals supplied from the same store and preparatory room. This might have been a chance coincidence but may as well have

been due to the dissimilar susceptibility or, probably, resistance of the other animals.

The infestation of the kangaroos was promoted by the working routine of the attendants, viz., that Monday rations (often also the fodder ration) were as a rule prepared on the preceding Friday afternoon. Thus a massive contamination of the rations with cat faeces could take place over the weekend during which also the freshly passed, non-infective oocysts may have undergone sporulation. This seems the more possible as derby-kangaroos have the habit of moving and eating during dusk and evening hours, so that they usually did not touch the Monday supply until the late hours of the afternoon and evening. Accordingly, at least 72 hours had elapsed between the preparation and ingestion of the Monday ration, a time long enough for the sporulation of *T. gondii* oocysts.

This again reminds of the importance of rigorous hygienic measures in Zoo animal management in general, and of the prevention of contamination of feed by cat faeces in particular.

It should be mentioned that our data concerning the onset of the disease may not have been accurate because, as already mentioned, derby-kangaroos usually keep quiet during the greater part of daytime and so the attendants may not have noticed the symptoms until they became quite severe.

This is the first report of the incidence of *Toxoplasma* infection among derby-kangaroos (*Thylogale(=Wallabia) eugenii*), which means that an additional species of the family *Macropodidae* are susceptible to this parasite.

## Summary

The death of three derby-kangaroos (*Thylogale* (= *Wallabia*) *eugenii*), kept in the same run in the Budapest Zoo, was found to have been caused by *Toxoplasma* infection. The diagnosis was established on the basis of histopathological examinations and experimental transmission to a young cat by feeding pieces of organs from the dead kangaroos. The cat, which had been kept in isolation before the infection experiment, began to discharge *T. gondii* oocysts a few days after ingestion of the organ pieces. Mice could be infected experimentally with sporulated oocysts collected from the faeces of the cat.

Investigations in the premises disclosed that the kangaroos became in all probability infected by the ingestion of fodder contaminated with cat faeces. Dry cat faeces found in the preparatory room and the fresh faeces of a cat permanently living there, both contained *Toxoplasma* oocysts, which caused fatal disease on experimental transmission to albino mice. The importance of rigorous hygienic measures in Zoo animal management and the importance of cats in the dissemination of *Toxoplasma* infection are pointed out.

The present paper is the first report of *Toxoplasma* infection in derby-kangaroos, new hosts of the parasites in the family *Macropodidae*.

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Address of the authors: Dr. Mihály DOBOS KOVÁCS, Dr. János MÉSZÁROS, Dr. László PELLÉRDY, 1400 Budapest, P. O. Box 2, Dr. András BALSAI, 1146 Budapest, Állatkerti út 8—10. Hungary

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# MUCOID ENTERITIS IN THE RABBIT ASSOCIATED WITH *E. COLI*

## CHANGES IN THE CARBOHYDRATE METABOLISM

By

F. VETÉSI and F. KUTAS

Department of Pathology (Head: Prof. A. KARDEVÁN) and Department of Physiology (Head: Prof. Gy. PETHES), University of Veterinary Science, Budapest

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Rabbits with mucoid enteritis develop hypotonic dehydration, decompensated metabolic acidosis and azotaemia (VETÉSI and KUTAS, 1973). The pathological changes can be attributed mainly to the effect of enterotoxin produced by enteropathogenic *E. coli* strains. Oral administration of *E. coli* enterotoxin provokes increased fluid and electrolyte secretion into the intestinal lumen of baby rabbits (SMITH, 1972; KUTAS et al., 1974). It has been supposed that toxic products such as endotoxins released by *E. coli* bacteria can be resorbed through the desquamated intestinal mucosa of rabbits with mucoid enteritis (VETÉSI, 1970). Consequent toxicosis associated with hypovolaemia due to fluid loss results in severe shock which causes the death of animal. Knowing the characteristic effects of bacterial endotoxins on carbohydrate metabolism (THOMAS, 1954; SANFORD et al., 1960), it seemed to be interesting to study the metabolism of carbohydrates in rabbits with mucoid enteritis. Unfortunately, little information is available on this mechanism, only KRUININGEN and WILLIAMS (1972) reported rise of blood sugar in affected rabbits, without any comment on the reason of hyperglycaemia.

Because of the lack of literary data, the more fundamental changes that occur at a cellular level need to be investigated in order to bring about a fuller understanding of metabolic disorders in mucoid enteritis. The present work was undertaken to study the intermediary steps of carbohydrate metabolism of affected rabbits with biochemical, histochemical and electron microscopic methods.

## Materials and Methods

A total of 39, weaned cross-bred ("Fehérgyöngy") rabbits with ages ranging from 4 to 11 weeks were used in this study. The material was almost identical with that used in our previous paper (VETÉSI and KUTAS, 1973). Of the 39 animals examined 23 developed naturally occurring form of mucoid enteritis and 16 served as healthy controls. The rabbits were fed a commercial granulated fattening diet *ad libitum*. In view of the diurnal rhythm of glycogen metabolism, the examinations were carried out always in the early afternoon hours. Procedures used for taking of samples, extermination of animals etc. have been described elsewhere (VETÉSI and KUTAS, 1973).

Level of blood sugar was determined by the o-toluidine method (Biochemica Test Combination, Boehringer Mannheim GMBH). Presence of urinary glucose was detected by Clinistix test (Ames Company).

Immediately after exsanguination, specimens were taken from the liver, kidney and heart muscle of 18 affected and 6 healthy rabbits. The specimens were fixed in absolute ethanol and in some cases in 8 per cent solution of buffered formalin. The tissues were embedded in paraffin and stained with periodic acid-Schiff reagent, with and without prior amylase treatment.

The histochemical study of the distribution of glucose-6-phosphatase in the cells of liver and kidney was made by the freezing method of CHIQUOINE (1953). Specimens were taken from 3 diseased and 2 control animals.

For the purpose of electron microscopic study, about 1 $mm^3$ fragments were cut from the liver of 3 diseased and 2 control rabbits. The samples were fixed in 2 per cent solution of osmium tetroxide buffered according to PALLADE. The fixation took place at +4°C temperature for 2 1/2 hours. The fixed specimens were dehydrated in alcohol-series, embedded in Durcupan-ACM-mixture and sectioned on a Reichert-OM-U2 ultramicrotome. The specimens were examined with a Tesla-BS 613 electron microscope.

## Results

The most characteristic clinical symptoms and post-mortem findings of rabbits with mucoid enteritis have been presented in a previous communication (VETÉSI and KUTAS, 1973).

Values for blood sugar of 19 affected and 14 healthy animals are shown in Table I. It can be seen that most of the blood sugar values of controls were found in a relatively narrow interval (91—120 mg/100 ml). In contrast, only 3 rabbits with mucoid enteritis had blood glucose concentration within these normal limits. The majority of affected rabbits (n = 12) were hyperglycaemic, in 4 other cases mild or severe form of hypoglycaemia could be demonstrated.

No glucose could be detected in the urine of 7 rabbits with mucoid enteritis, although 5 of them were hyperglycaemic. The urine of the control animals (n = 5) did not contain glucose either.

Besides the determination of the blood sugar, the glycogen stores of the organism were also studied. On the basis of the histochemical investigations it could be established that the distribution of glycogen granules appeared normal in the hepatocytes of the healthy controls (Fig. 1). The glucose-6-phosphatase enzyme was also unformly distributed in the parenchymal cells of liver.

In contrast to the healthy rabbits, a characteristic decrease of glycogen content was observed in the liver of affected animals. In a few instances, the

**Table I**

Blood sugar levels in control rabbits and rabbits with mucoid enteritis

| Characterization of condition | Blood sugar (mg/100 ml) | | Number of rabbits |
|---|---|---|---|
| | Range | Individual values | |
| Hypoglycaemia, severe | 0—40 | 40, 36 | 2 |
| Hypoglycaemia, mild | 71—90 | 71, 73 | 2 |
| Normal | 91—120 | 97, 117 | 2 |
| | | 92,* 95,* 96,* 97*, 98*, 99,* 103,* 111,* 111,* 114,* 116*, 118,* 118* | 13* |
| | 121—140 | 124 | 1 |
| | | 131* | 1* |
| Hyperglycaemia, mild | 141—200 | 144, 183, 187 | 3 |
| Hyperglycaemia, severe | 201—300 | 207, 219, 248, 254, 277, | 5 |
| | 301—400 | 310, 317, 339, 369 | 4 |

* Control animals

glycogen granules disappeared only in the periphery of the lobules, near by the periportal zone (Fig. 2). In most cases, there was no demonstrable glycogen present in the hepatocytes of the intermedial zone either. Only the cells around the central vein contained glycogen granules (Fig. 3). Higher activity of he-

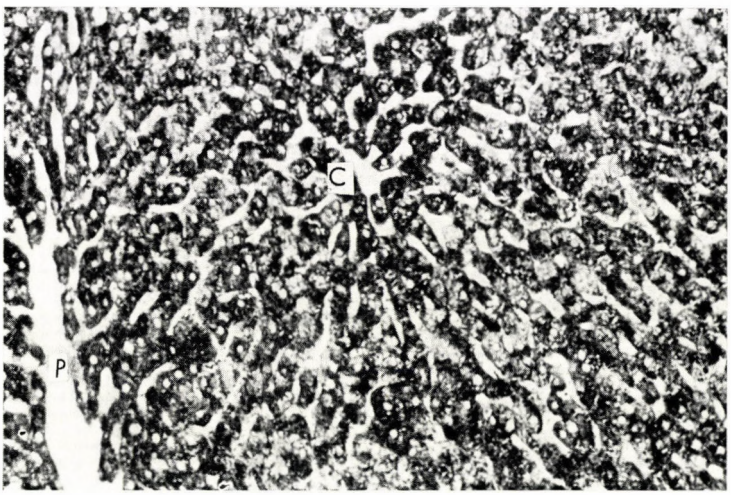


*Fig. 1.* Liver of control rabbit. Abundant PAS-positive glycogen granules in the hepatocytes; c, central vein; p, periportal zone; ×110 approximately

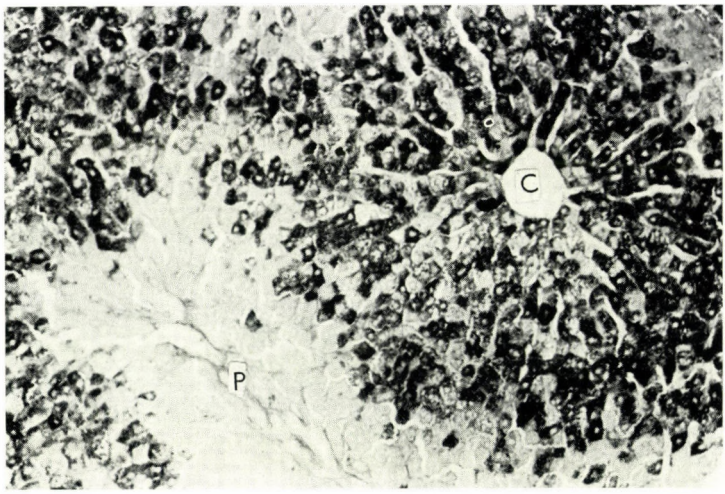


*Fig. 2.* Liver of rabbit with mucoid enteritis. Cells adjacent to the periportal zone (p) contain no glycogen granules. PAS reaction; c, central vein; ×110 approximately

patic glucose-6-phosphatase was found in the periphery of the lobules than in the centrilobular zone (Fig. 4).

Electron micrographs of the liver of rabbits with mucoid enteritis corroborated the significant decrease in cellular glycogen content. Hypertrophy of the smooth and to a certain extent, of the rough endoplasmic reticulum was observed (Figs 5 and 6).

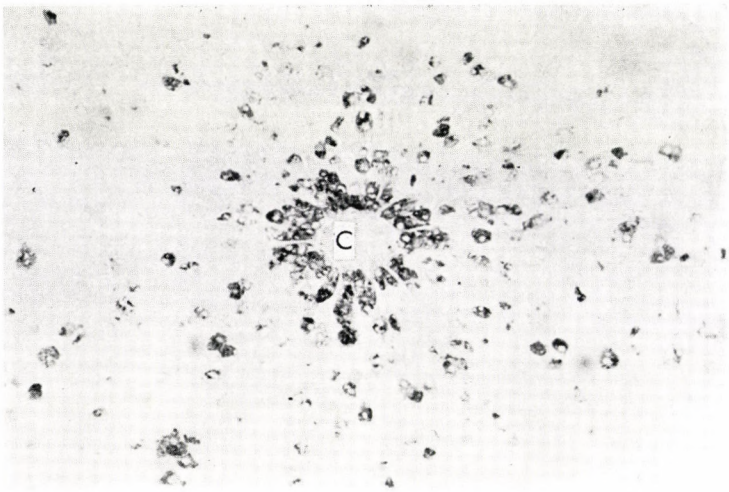


*Fig. 3.* Liver of rabbit with mucoid enteritis. Depletion of glycogen. PAS-positive glycogen granules are only around the central vein (c); ×110 approximately

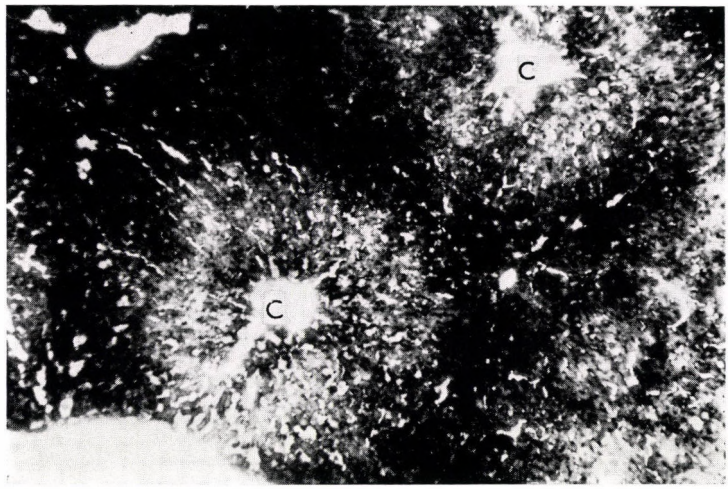


*Fig. 4.* Liver of rabbit with mucoid enteritis. Higher activity of glucose-6-phosphatase in the periphery of the lobules; c, central vein; ×40 approximately

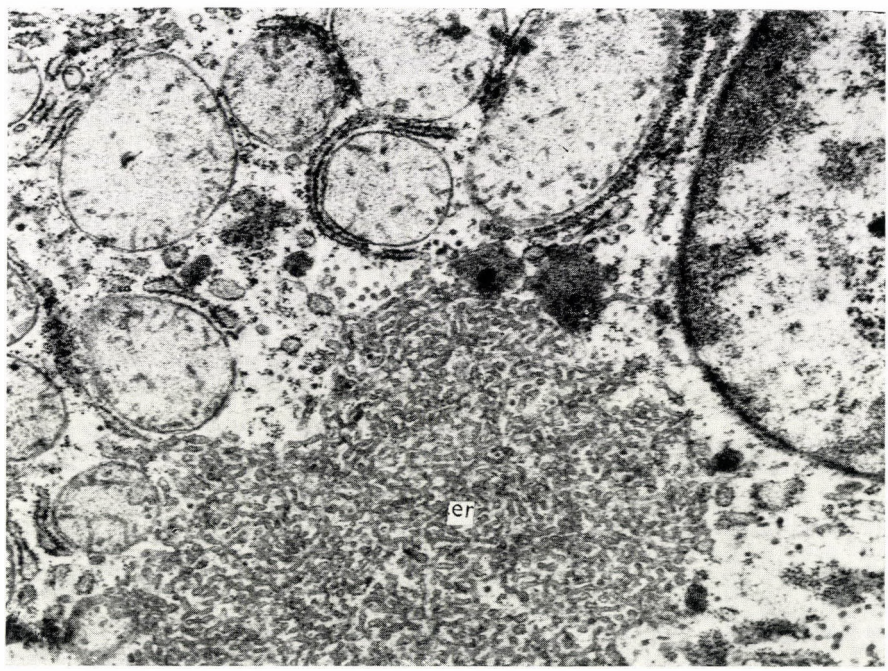


*Fig. 5.* Liver of rabbit with mucoid enteritis. Slight hypertrophy of the endoplasmic reticulum (er) in the hepatocyte. Electron micrograph; ×17,820

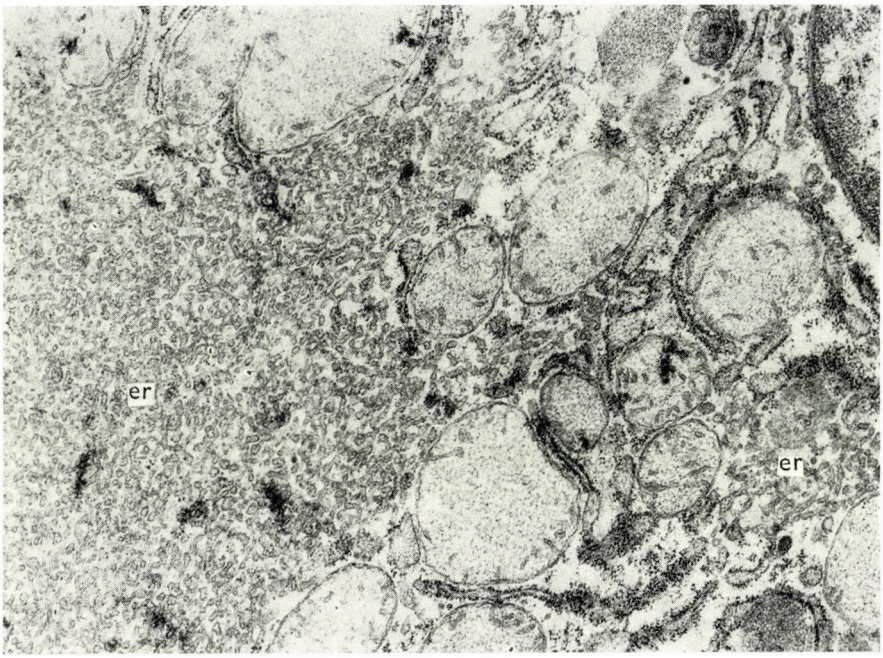


*Fig. 6.* Liver of rabbit with mucoid enteritis. Hypertrophy of the endoplasmic reticulum (er) in diffuse form. Electron micrograph; ×17,820

Histochemical examination of myocardial fibres of affected rabbits showed also reduction in glycogen. In some cases, the disappearance of glycogen was so advanced that only some fibres contained glycogen granules.

The brush border in the proximal renal tubules of the affected rabbits was more intensively stained with periodic acid-Schiff reagent than that in control animals. In some rabbits with mucoid enteritis, differently from the controls, PAS-positivity stained, fine granulates and larger corpuscles could be observed in the epithel cells of the convoluted tubules. Following amylase treatment, the brush border and the larger corpuscles were invariably stained with PAS reagent, while the finely granulated material disappeared and no PAS-positivity could be detected.

The activity of glucose-6-phosphatase was higher in the renal cortex of diseased rabbits than in that of controls.

## Discussion

From the present studies it can be established that mucoid enteritis caused breakdown of hepatic and heart muscle glycogen to glucose, con-

sequently hyperglycaemia developed. However, at the terminal stage of disease when the glycogen stores were depleted, the blood sugar began to fall toward hypoglycaemic levels.

A greatly increased hepatic glucose-6-phosphatase activity could be observed especially in the periphery of the lobules where the most active form of glycogenolysis was detected. This enzyme is essential in the glycogenolytic action of liver and kidney, cleaving glucose-6-phosphate to free glucose and phosphate. At the same time, it was characteristic the hypertrophy of the endoplasmic reticulum where the glucose-6-phosphatase enzyme is localized (ERICSSON, 1966; MÜLLER, 1971). The endoplasmic reticulum is known as the main site of the hepatic glycogenolysis (MÜLLER et al., 1966).

As shown in the present paper, an excessive accumulation of glycogen was observed histologically in the proximal tubules of kidney. Studies in alloxan-diabetic rats showed also glycogen deposition in the proximal tubules (CURTIS et al., 1947). Thirty-fold higher level of glycogen was found in the renal cortex of diabetic rats than in that of controls (ANDERSON and STOWRING, 1973). The accumulation of glycogen in the kidney could be explained with the increased rate of renal gluconeogenesis. The diabetic state was accompanied by depletion of hepatic glycogen, hyperglycaemia and glycosuria. Thus, the metabolic changes of carbohydrate balance were similar to those observed on rabbits in the present study. However, a characteristic difference was that the urine of hyperglycaemic rabbits with mucoid enteritis did not contain glucose. The aglycosuria may be in relationship with the circulatory failure of the shocked rabbit. In this condition hypotension develops and urine formation ceases when the arterial pressure falls below 60 to 80 mmHg (BLAND, 1956). The urinary bladder of the affected rabbits was empty or contained only 1—2 ml of urine, indicating clinical signs of anuria or oliguria (VETÉSI and KUTAS, 1973). There is a further explanation for the aglycosuria, with regard to the findings of FÖLDI et al. (1948). The authors observed passive rediffusion of glucose through the degenerated tubular epithelium. Since rabbits with mucoid enteritis develop tubulonephrosis, it is possible that the unabsorbed glucose of glomerular filtrate diffuses back into the bloodstream.

The exact mechanism and reason of the increase of blood sugar are not clear. According to the present opinion, hyperglycaemia associated with endotoxin shock is due, at least in part, to adrenaline released from the adrenal medulla. SANFORD et al. (1960) observed that adrenalectomy, with cortisol replacement, prevented the hepatic glycogenolysis produced by the injection of endotoxin into rats. BENEDECZKY and BERTÓK (1968) described intensive disappearance of catecholamine granules in the adrenal gland of endotoxin treated rats. One of us (VETÉSI, 1974) found similar phenomenon in adult rabbits after injection of *E. coli* endotoxin. One the other hand, KERTAI

(1972) could not produce hyperglycaemia in depancreatized and insulin treated dogs following endotoxin injection. This observation suggests the possible role of pancreatic glucagon hormone in the mechanism of hyperglycaemia. Both adrenaline and glucagon act similarly: the hormones activate hepatic phosphorylase via the adenyl cyclase–cyclic AMP system. Evidence has been obtained to indicate that the rise in adenyl cyclase activity is one of the earliest cytotoxic effect in endotoxin shock (JACOBSON et al., 1972).

In addition to the effect of endotoxin, it should be taken into consideration that hypoxia and acidosis described in rabbits with mucoid enteritis (VETÉSI and KUTAS, 1973) may be also responsible for stimulated glycogenolysis. It is known that hypoxia causes rapid breakdown of liver and muscle glycogen. Under anaerobic conditions, the carbohydrates are decomposed via the *Embden—Meyerhof* glycolytic pathway with a less efficient rate of ATP formation. Since an adequate supply of oxygen is necessary for glycogen synthesis, the glycogen stores are soon depleted. Furthermore, severe acidosis due to faecal bicarbonate loss also stimulates glycogenolysis. ELIAS (1912) showed that intravenous injection of acid into dogs reduced hepatic glycogen content and caused hyperglycaemia.

After all, it can be established that due to several glycogenolytic factors, a sharp reduction in the cellular glycogen content occurs. Since glycogen is the main intracellular substrate as energy source, with progression of shock, there may be a decrease in the energy production of cells. Considering the vital function of the organ, the fall in the heart glycogen level seems to be especially deleterious.

The results of the present investigation suggest that depletion of glycogen stores may account in large part for the development of the irreversibility in shock caused by mucoid enteritis.

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## Summary

The effect of naturally occurring mucoid enteritis on the carbohydrate metabolism was investigated with biochemical, histochemical and electron microscopic methods. Besides the examination of affected rabbits, studies were also performed on healthy animals of the same herd.

The carbohydrate metabolism of rabbits with mucoid enteritis can be described as follows: increased glycogenolysis in liver and heart muscle, high activity of hepatic and renal glucose-6-phosphatase, aglycosuric hyperglycaemia, accumulation of glycogen in the proximal tubules of kidney.

The authors suggest that severe disturbances in carbohydrate metabolism, first of all the depletion of cellular glycogen stores, may account in large part for the fatal end of disease.

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Address of the authors: Dr. Ferenc VETÉSI, Dr. Ferenc KUTAS, 1078 Budapest, Landler J. u. 2, Hungary

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# STUDIES ON THE PATHOGENICITY OF *TOXOPLASMA* FOR MICE

By

L. PELLÉRDY and M. DOBOS-KOVÁCS

Department of General Zoology and Parasitology (Head: Prof. T. KOBULEJ) and Department of Pathological Anatomy (Head: Prof. A. KARDEVÁN), University of Veterinary Science, Budapest

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The pathogenicity of the vegetative form of *Toxoplasma* for humans and various animals has been elucidated in great detail by extensive investigations conducted since the beginning of this century. Little, is however, known about the effects of *Toxoplasma gondii* oocysts, passed by the cat and sporulating in the outside world, on non-feline hosts after taking them up orally.

FRENKEL (1973) made in his textbook a brief reference to oocyst pathogenicity, illustrating it with a few histological pictures, but not explaining it in a greater detail. More information is emerging from the report of DUBEY and FRENKEL (1973), who concluded from studies on four *Toxoplasma* strains that orally administered oocysts were definitely pathogenic for mice, although the degree of damage varied with the strain. Two strains caused a lethal toxoplasmosis in the mouse on oral administration of very few — 1 to 10 — oocysts, whereas the other two required a massive dose — at least 1000 oocysts — to produce the same effect. Systematic investigations into the pathogenicity of *Toxoplasma gondii* oocysts for mice have not yet been reported.

The present studies were carried out with the aim to throw more light on the problem by infection experiments and detailed analysis of gross and microscopic lesions.

## Materials and Methods

Part of the oocysts used in the studies were isolated from young cats in this laboratory (strain "Budapest"), part belonged to the strain "Gail" received through the courtesy of Professor PIEKARSKI (Bonn, GFR).

Both strains were maintained by serial transfers in mice as follows: organ suspensions prepared from cyst-containing mouse brain in saline were administered orally through a gastric tube to susceptible mice. Those surviving the infection developed *Toxoplasma* cysts in the brain after a certain time.

The shortest interval between transfers from mouse to mouse was 15 days, the longest 187 days in our hands. Administration of the brain suspension to the recipient was always carried out per os and within one hour after the

extermination of the donor. Prior to transfer, the presence of cysts in the infectious material was ascertained by microscopic examination.

To obtain oocysts from cats, first the *Toxoplasma* infected mouse brain, secondly the entire mouse was fed to cats of various ages. Part of the cats began to pass oocysts after one week; these were, after an appropriate enrichment and purification, sporulated in a 2% aqueous potassium bichromate solution at room temperature, washed several times in water by centrifugation and stored at +4°C until used. The oocysts were counted in a Buerker-type haemacytometer and the results were compared with those obtained by the traditional simple method of parasite egg and oocyst counting, *viz.*, oocysts were counted in 20—25 fields of vision under a coverslip placed on 0.05 ml suspension, the average number of oocysts for one field of vision was calculated and multiplied by the number of fields in the entire area of the coverslip and the result was multiplied by 20 to obtain the total number for one ml. The counts determined by the two procedures were always in good accordance with each other.

For mouse pathogenicity studies, oocysts of both strains were obtained from cats as described above and were administered orally to groups of albino mice. The individual dose range was five oocysts to one million. Mice killed by the toxoplasma infection were processed either immediately or 1—2 hours after death at the latest. Specimens secured from various organs (several segments of small intestine, mesenteric lymph nodes, liver, spleen, kidneys, pancreas, lungs, heart, brain, *etc.*) for histological examination were fixed in 10% formalin, embedded in paraffin, and the sections were stained with haematoxylin and eosin.

In three experimental groups, isolation of bacteria was attempted from various segments of small and large intestine and liver, under aerobic conditions, on common and blood agar as well as on Drigalski's medium.

Appropriate control mice of the same age and breed and maintained under identical conditions were set up with each experimental group.

## Experimental

a. *Isolation of the "Budapest" strain.* About two years ago a young cat examined was found to pass with the stool large amounts of oocysts, which appeared to correspond to the type formerly known as *Isospora bigemina.* They were round or elliptic, and 11—15 by 9—12 $\mu$m in size. The oocysts were sporulated in the laboratory and administered to 10 mice, which all died between 5 and 11 days after infection. The infection experiment was repeated on three additional occasions, again on groups of 10 mice, using 50,000 to 100,000 oocysts as individual dose. The infection was lethal in every instance.

Out of 10 mice infected about one and a half months later two had a protracted course of disease: one died on the 13th day after infection, the other was killed in the terminal stage simultaneously. These two mice had *Toxoplasma* cysts in the brain, which were never found in mice died earlier. The strain could be maintained by oral transfer of the cyst-containing brain material to susceptible mice.

Several biological properties of the isolate were compared with those of the strain "Gail" and the two strains were found to be essentially similar. No morphological differentiation was possible on the basis of brain cysts and oocysts passed by the cat and no notable difference was found either in mouse pathogenicity.

**Table I**

Pathogenicity of cysts of various ages of the *Toxoplasma* strains "Budapest" and "Gail" on oral administration to the mouse

| Latency period (days) | Strain Gail | Strain Budapest |
|---|---|---|
| | Number of mice died within 14 days/number of mice infected | |
| 15 | 3/10 | |
| 19 | | 1/10 |
| 20 | | 0/10 |
| 22 | | 2/10 |
| 23 | 0/10 | 0/10 |
| 35 | 7/10 | |
| 37 | | 1/10 |
| 47 | 1/10 | |
| 70 | 5/10 | 2/10 |
| 77 | | 2/10 |
| 106 | 7/10 | 5/10 |
| 187 | 5/10 | 3/10 |

b. *Pathogenicity of Toxoplasma cysts.* Oral infection with cyst-containing brain material killed the majority of the mice within 14 days, the minority survived. The mortality was still higher and the symptoms were more severe when brains of mice containing older cysts after a long-standing infection were used. In such cases losses often reached 70%, whereas with young cysts the maximum mortality was 30% (Table I). The higher pathogenicity of old cysts may have been due to the greater number of bradyzoites inside them or, probably, to the development of futher cysts from the released bradyzoites.*

* The term "bradyzoites", proposed by FRENKEL (1973), is used to designate the apparently resting developmental stages formerly designated as zoites or merozoites, which localize inside the cysts, as opposed to the vigorously multiplying tachyzoites (formerly called trophozoites, proliferative stages, *etc.*), which occur single, in pairs or in groups of various sizes in different organs.

The observation that some of the surviving mice unexpectedly began to show rotating movements after a relatively long symptomless period, weighs in favour of this implication. The rotating movements, lasting a few days, may well have been the sign of development of new cysts in the brain. This accords well with the microscopic finding that apart from the usual cysts, 40—50 $\mu$m or even more in diameter, the brains of mice with a long-standing toxoplasmosis often contained smaller forms as well (Fig. 1).

c. *Pathogenicity of Toxoplasma oocysts.* Experimental infection with sporulated oocysts of the "Budapest" and "Gail" strains killed almost all mice,

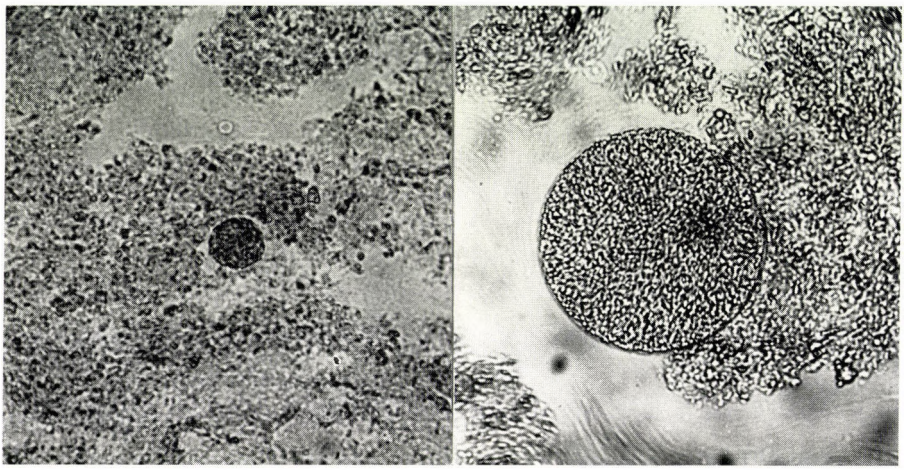

*Fig. 1. Toxoplasma* cysts of various sizes in the brain of one and the same mouse, about two months after oral infection with cyst-containing brain material. Unstained preparation; × about 750

irrespective of their age, within 5—11 days. Survival was exceptional and oocyst infection could well be regarded as lethal for mice. The two strains did not notably differ in respect of pathogenicity.

Investigations into the oocyst dose required for pathogenic effect led to the surprising discovery that neither severity of the infection nor death seems to depend much on the actual number of orally administered oocysts (Table II). As in the previous experiments individual doses of $10^5$—$10^6$ oocysts proved to be 100% lethal for mice, only doses of 50,000 or less oocysts were tested for pathogenicity in the comparative experiments. As seen in Table II, mice given the larger doses usually died earlier than those fed with the smaller ones. There were, however, several exceptions, *e.g.*, the first mouse in the group treated with 5000 oocysts already died on the sixth day, whereas among mice given 50,000 oocysts, losses began to occur one day later. Mice given the lower doses

**Table II**

Pathogenicity of oocysts of the strain "Budapest" on oral administration to mice

| Number of oocysts used for infection | 50,000 | | | 5,000 | | | 500 | | | 50 | | | 5 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No. of experiments | I | II | III | I | II | III | I | II | III | I | II | III | I |
| No. of mice | 4 | 3 | 3 | 4 | 3 | 3 | 4 | 3 | 3 | 4 | 3 | 3 | 4 |
| 6. day p.i. | | | | | + | | | | | | | | |
| 7. day p.i | ++<br>++ | | ++ | | | | | | | | | | |
| 8. day p.i. | | ++ | | | + | + | | | ++ | | + | + | |
| 9. day p.i. | | | | ++<br>+ | + | | ++ | + | | ++<br>+ | ++ | | + |
| 10. day p.i. | | + | + | | | + | | ++ | | | | | |
| 11. day p.i. | | | | | | + | ++ | | + | | | ++ | ++<br>+ |
| Survivors | | | | + | | | | | | + | | | |

p.i. = post infection

usually died between 9 and 11 days after infection, whereas in the large-dose groups very few animals survived the ninth day.

d. *Clinical observations.* Mice infected orally with oocysts of strain "Budapest" or strain "Gail" became listless a few hours before death. Their hair was ruffled and dirty and the skin of their tail and ear assumed a bluish-grey tint. Part of the diseased mice developed diarrhoea, passing instead of the normal pellets a pasty or watery stool admixing with mucus. In some cases practically the entire faeces consisted of a viscous mucus, forming occasionally a cylindrical clot. The perianal hair was glued together by the mucous faeces, which soiled the hair also in other parts and the cylindrical clot of mucus often adhered to the root of the tail. It should be mentioned that the other part of the animals did not show any sign of diarrhoea, although otherwise they were severely ill.

The terminal stage was characterized by weakness of the hind part of the body, greater or lesser increase of abdominal volume, slow, swaying movements and, finally, complete prostration which was followed by death within seconds or minutes.

e. *Gross lesions.* In a few carcases the spleen was swollen, but in the majority it appeared normal. A portion of the small intestine was distended, above all the ileum, but occasionally also some segments orally to the ileum, showed

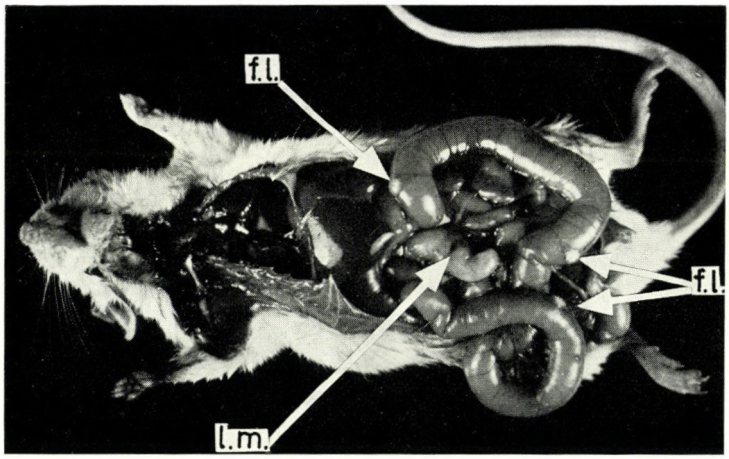


*Fig. 2.* Intestine of mice died after oral infection with oocysts. Note distension of the small intestine and enlargement of intestinal lymphatic follicles (f.l.) and mesenteric lymph nodes (l.m.). Roughly natural size

distension over a greater or lesser distance (Fig. 2). The wall of the small intestine appeared either dark red, or greyish-white or greyish-yellow when viewed through the serosa, and it was always glossy, owing to serous infiltration. The distended portion of the small intestine was filled by transparent slightly opalescent or turbin contents, which were either watery or mucous and viscous, infrequently jellinaceous in consistency. There was a distinct mural thickening in the affected portions. The contents of the large intestines rarely

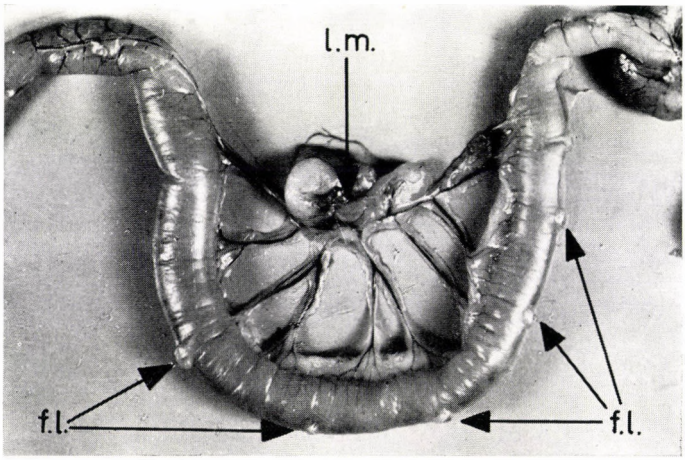


*Fig. 3.* Segment from small intestine of a mouse died after oral infection with oocysts. The intestine is distended, the enlarged intestinal lymph follicles (f.l.) are conspicuous and an enlarged mesenteric lymph node (l.m.) is seen in the centre. About double life-size

resembled those of the small intestine, but usually they were either pasty or normal. The mucous membranes of both small and large intestines were greyish-white, less often dark red in colour, but always appeared swollen and thickened.

The lymphatic follicles of the small intestine enlarged to the size of a millet seed and, protruding beneath the serosa, could easily be distinguished from the darker intestinal wall by their greyish-white colour (Fig. 3). In cer-

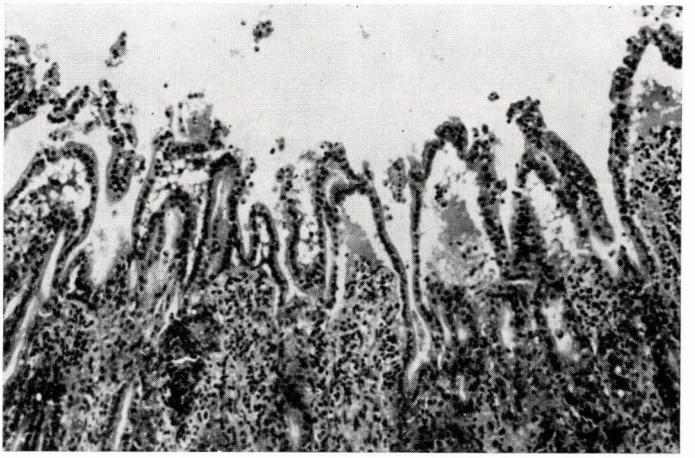

*Fig. 4.* Section from small intestine of a mouse died after oral infection with oocysts. Subepithelial plasma deposition lifts the mucous endothelium. HE stain; × about 110

tain cases greyish-red eroded areas, showing the contours of lymphatic follicles were found in the mucosa corresponding to the follicles.

The mesenteric lymph nodes enlarged to different degrees (sometimes enormously), assumed a greyish-white colour and showed, together with the surrounding mesenterium, a serous infiltration (Figs 2—3). Upon incision, glossy, juicy areas of indistinct structure alternated with nacreous, pin-prick foci.

The liver usually appeared normal, in a few cases it was slightly enlarged, greyish-brown in colour and rigid. The surface of its incisions showed loss of structural definition and sometimes a few greyish-white, pin-prick foci were seen on them. The parenchyma was friable. The kidneys were normal.

The lungs showed hyperaemia and acute emphysema of varying severity, but no other lesion. In a few cases there was a general, acute dilatation of the heart.

f. *Microscopic lesions.* In various segments of the small intestine, serum like fluid staining homogeneously with eosin separated the mucous epithelium from the propria (Fig. 4). The epithelial coat of the intestinal villi was shed

in large patches in several places, the shreds admixing with the contents of the intestinal lumen (Fig. 5). Occasionally the epithelial desquamation was limited to the apical parts of the villi.

The cytoplasm of most epithelial cells was loose and vacuolized. Cells in the process of desquamation or already shed showed nuclear pycnosis and rhexis. Developmental stages of toxoplasms could not be detected in the intestinal epithelial cells. In certain cases many bacteria were found at the

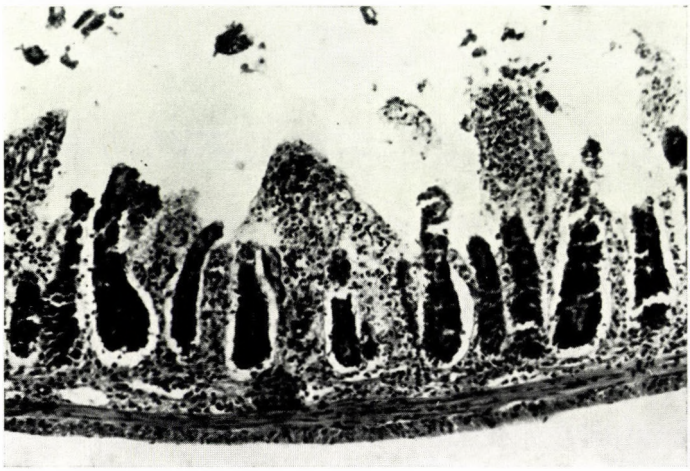

*Fig. 5.* Complete desquamation of the epithelial layer in small intestine. HE stain; × about 110

luminal side of intestinal epithelial cells and, after their desquamation, on the surface of the denuded propria.

The propria of the mucosa, including the stratum villosum of the villi, showed an accumulation of fluid and loss of structural definition. In places, infiltrating neutrophilic granulocytes and residues of deteriorated cells (macrophages) were also seen in it (Fig. 6). Complete destruction of the mucosal glands occurred in several parts of the denuded areas. In certain intestinal segments only the connective tissue of the propria and inside the fibrous network residues of deteriorated cells were left. The mucosal vessels were usually markedly distended and filled with erythrocytes. Lymph spaces and lymph vessels in the intestinal wall were as a rule distended and filled by lymph containing various numbers of cells.

The intracellular stages of toxoplasms (minor or major groups of tachyzoites) usually localized in the cytoplasm of macrophages (reticulum cells), less often in other cells (*e.g.*, neutrophilic granulocytes) of the propria. In certain places single stages were encountered in an extracellular localization (Fig. 7). Tachyzoites were found in the muscularis mucosae, occasionally also

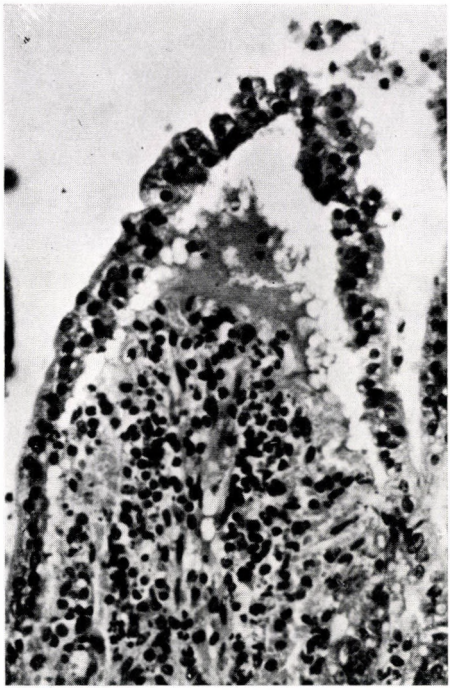

*Fig. 6.* The mucous epithelium of the small intestine is lifted from the intestinal villus by accumulation of homogeneously staining fluid (serum). The villous layer of the villus contains many cell debris, proliferating reticulum cells and neutrophilic granulocytes. HE stain; × about 275

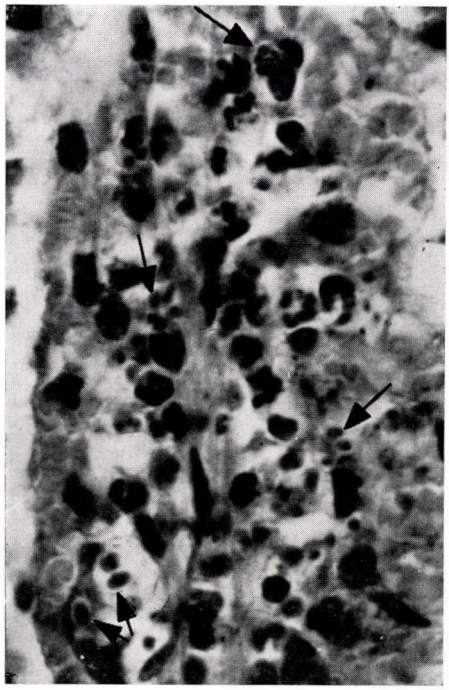

*Fig. 7.* Released tachyzoites (arrows) in the propria of the small intestine. HE stain; × about 700

in the submucosa, occurring sometimes in groups inside the cytoplasm of macrophages. Neither the muscle layer, nor the serous membrane of the intestine showed notable lesions.

Extracellularly localizing single tachyzoites and groups of tachyzoites inside the macrophages were found in the lymph spaces, lymph capillaries and minor lymph channels of the intestinal villi (Fig. 8).

In the intestinal lymphatic follicles the wide lymph spaces were filled by a fluid rich in cells and cell debris. Groups of various numbers of tachyzoites occurred inside the reticulum cells forming the basic structure of lymphatic follicles (Fig. 9). The reticulum cells showed a vigorous phagocytic and proliferative activity.

The mesenteric lymph nodes showed various degrees of reticulum cell hyperplasia and the cells themselves displayed a vigorous phagocytic activity. The distended sinuses were filled by lymph, containing desquamated cells of sinusoidal endothelium and cell debris. The desquamated endothelial cells



*Fig. 8.* Group of tachyzoites inside a reticulum cell localizing in the lumen of a distended mural lymph capillary in the small intestine. HE stain; × about 1800

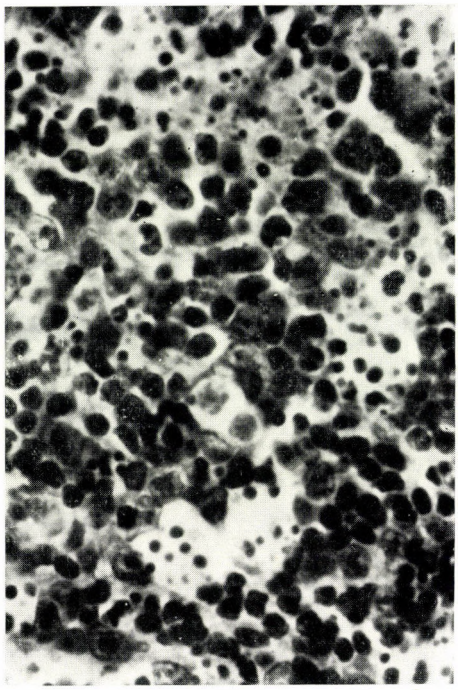

*Fig. 9.* Intestinal lymph follicle. The lymph spaces are distended and filled by lymph usually containing cell debris. Groups of tachyzoites are seen inside a few reticulum cells. HE stain; × about 700

and the reticulum cells of the supporting structure contained one or more tachyzoites (Fig. 10).

The liver cells were in the state of cloudy swelling and the cells of the RHS system showed various degrees of focal or diffuse proliferation. Groups of tachyzoites were sometimes found in proliferating cells (Fig. 11).

The splenic follicles were slightly enlarged and their reticulum cell elements showed a vigorous phagocytic activity. Part of the reticulum cells had fragmented nuclei, others were shrunken, and some of them were proliferating. No *Toxoplasma* stages were found in the spleen, only a few structures reminiscent of them.

In the kidneys, the secondary contorted tubules showed tubulonephrosis of varying severity.

No notable microscopic lesions were found in the pancreas, heart and lungs. The brains of those mice which died within 5—11 days after infection neither contained *Toxoplasma* cysts, nor showed changes related to the presence of *Toxoplasma*.

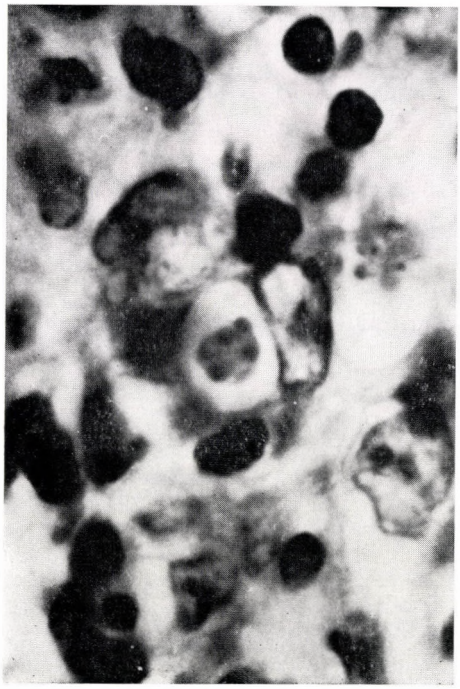

*Fig. 10.* Group of tachyzoites surrounded by a parasitophorous vacuole inside a reticulum cell of a mesenteric lymph node. HE stain; × about 1800

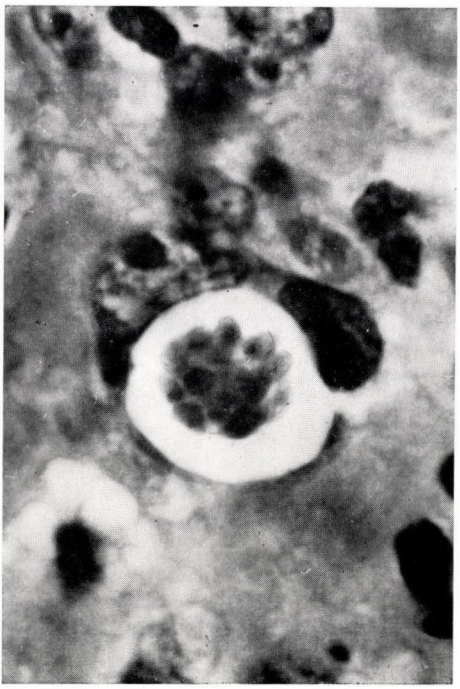

*Fig. 11.* Group of tachyzoites in a proliferating hepatic RHS cell. HE stain; × about 1800

In the brains of the survivors, cysts and intracellularly localizing groups of tachyzoites were found from the 13th day on. The establishment of the parasites in the brain was accompanied by histio-lymphocytic infiltration along some meningeal and cerebral vessels and focal proliferation of glia cells in the brain. Toxoplasma stages were found in various parts of the brain. By the time the stages established themselves in the brain, little if any indication was found of the organic lesions described in the foregoing.

g. *Bacteriological examinations.* Isolation of bacteria was attempted from the intestinal contents and livers of mice from three experimental groups, infected with different oral doses of oocysts. In every case, pure cultures of *Escherichia coli* bacteria were isolated in great number from various segments of the small intestine and from the caecum and colon. Mice examined bacteriologically before oral infection with oocysts yielded no bacteria in the contents of the anterior third or half of the small intestine, whereas in the posterior part as well as in the caecum and colon only very few *E. coli* organisms were present among the other flora components (*e.g.*, enterococci).

Occasionally a few colonies of *E. coli* could be isolated from the liver of oocyst-infected mice, but in the majority of the cases the liver was bacteriologically negative.

The aqueous suspension of *Toxoplasma gondii* oocysts used for the experimental infections proved to be negative when checked bacteriologically.

## Discussion

According to the present studies, the feline *Toxoplasma gondii* strains "Budapest" and "Gail" are very similar in respect of morphological characteristics and pathogenicity for the mouse.

In infection experiments, oocysts passed from the digestive tract of the cat caused a severe, usually lethal disease in both young and old albino mice on oral administration. In certain cases five oocysts were sufficient to kill the mouse after a period of illness, but as a rule, pathogenicity was more pronounced with larger oocyst doses.

Microscopic examination revealed developmental stages of *T. gondii* in various organs of those mice which succumbed to oral infection with oocysts. Various numbers of stages were found in the villous epithelium of intestinal villi, in deeper layers of the propria, in intestinal lymph follicles and mesenteric lymph nodes and, infrequently, in the liver parenchyma. Single tachyzoites or groups of them chiefly occurred in reticulum cells, less often in other cells (*e.g.*, in neutrophilic granulocytes and endothelial cells of lymph sinuses). The vigorous intracellular multiplication of tachyzoites injures and kills many cells. The pronounced phagocytic activity of the RHS cells in various organs (lymphatic follicles of small intestine, mesenteric lymph nodes, liver and spleen) plays an important role in the destruction of the parasites.

No cysts were found in the brain of those mice which died within 11 days after experimental infection. In survivors cysts appeared 13 days after infection at the earliest.

Lesions indicative of myocarditis, pneumonitis and encephalitis, as reported by DUBEY and FRENKEL (1973) were not found by us. A focal encephalitis, which was in all probability related to *Toxoplasma* infection was nevertheless found in those animals which had survived the 11th day and were killed at a later time.

Oral infection with cysts in brain suspension proved to be less severe than oocyst infection: a greater or lesser part of the infected mice survived and bradyzoites developed inside the cysts in their brain. The greater mortality observed on using brain material from mice with a long-standing toxoplasmosis may have been due to the multiplication of *Toxoplasma* cysts in the brain of the donors.

The surprising observation that very few oocysts could produce a lethal illness, although death ensued somewhat later than with massive doses, has led us to speculate whether the fatal outcome was in fact due primarily to the establishment of the parasites in the host followed by cell destruction by the developmental stages and, probably, by the production of the hypothetical toxotoxin. The symptoms (mucous stool, severe peripheral circulation insufficiency), postmortem findings (accumulation of fluid in the intestinal lumen, serous infiltration of the intestinal wall) and, especially, the microscopic lesions (desquamation of epithelium owing to subepithelial plasma accumulation in the small intestine, regressive lesions of epithelial cells, distension and congestion of blood and lymph vessels with blood and cellular lymph, respectively, tubular nephrosis, *etc.*) suggested that the death was caused by shock. The experimental observations indicate that the shock was elicited by a toxic effect, *viz.*, an enterotoxaemia, in which *E. coli* bacteria also played a role, to judge from their marked increase in certain intestinal segments of the sick animals. The explanation lies close at hand that *Toxoplasma* invasion brings about various functional disorders in the intestine, one of which is manifested by the exalted growth of *E. coli*, a component of the normal flora. The considerable amount of toxin (endo- and enterotoxins), released by the greatly multiplied *E. coli* organisms may, in addition to the direct effect of toxoplasms, play a decisive role in the development of enterotoxaemia, *viz.*, of acute endotoxin shock in mice orally infected with oocysts. The intestinal changes found by us in fatal cases of experimental murine toxoplasmosis were similar to those observed by BERTÓK and TÓTH (1963) in mice treated intravenously and intraperitoneally with the LPS-fraction of *E. coli* endotoxin, and even to those found in rabbits with the mucous enteritis generally attributed to enterotoxaemia (VETÉSI, 1969). It is known that the development of enterotoxaemia is promoted by predisposing factors, *e.g.*, in rabbits the mucosal injuries and functional disorders caused by intestinal coccidiosis may greatly favour the establishment of mucous enteritis (LÖLIGER et al., 1969). We are therefore of the opinion that in mice infected orally with *T. gondii* oocysts, enterotoxaemia, *viz.*, the toxin shock related to the extreme multiplication of *E. coli* in the intestinal contents, plays a decisive role in the fatal outcome of the disease.

## Summary

A *Toxoplasma* strain designated "Budapest" was isolated from a cat of unknown origin by oral administration of the sporulated oocysts to mice. The strain was maintained by oral transfer of mouse brains containing cysts to mice at intervals ranging from 15 to 187 days. The mouse pathogenicity of the strain "Budapest" was similar to that of the strain "Gail", isolated in Bonn (GFR). The majority of the mice infected orally with oocysts discharged by the cat died within 5—11 days and even doses as low as 5 oocysts were sufficient for a lethal effect.

Postmortem examinations showed distension of, and accumulation of fluid in, the small intestine, serous infiltration of the intestinal wall, signs indicative of peripheral circulation in-

sufficiency, enlargement of intestinal lymphatic follicles and mesenteric lymph nodes and extreme multiplication of *E. coli* in certain intestinal segments. Microscopic examination revealed the presence of developmental stages of *Toxoplasma* in various organs (intestinal mucosa, intestinal lymphatic follicles, mesenteric lymph nodes and, occasionally, liver).

Most gross and microscopic lesions strongly suggested that the death of the mice infected with *Toxoplasma* oocysts was caused by an acute shock. Bacteriological examinations of the intestinal contents indicated that an exalted growth of *E. coli* bacteria and a consequent enterotoxaemia plays an important role in the development of lethal shock in mice orally infected with *Toxoplasma* oocysts.

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Address of the authors: Dr. László PELLÉRDY, Dr. Mihály DOBOS-KOVÁCS, 1078 Budapest, Landler J. u. 2, Hungary

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# PNEUMOENTERITIS OF LAMBS CAUSED BY ADENOVIRUS

## PRELIMINARY REPORT

By

S. Belák and V. Pálfi

Department of Epizootiology (Head: Prof. J. Mészáros), University of Veterinary Science and the Central Veterinary Institute (Head: A. Ványi), Budapest

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Successful isolation of adenoviruses from sheep was reported only by McFerran et al. (1969, 1971) so far. The aetiological role of these viruses has not been studied yet. Recently we have also succeeded in the isolation of an adenovirus from lambs showing respiratory symptoms. The virus, designated Het/3 proved to be closely related to type 2 of bovine adenoviruses and was found to be pathogenic for both lambs and calf. These findings will be soon published elsewhere. However, more recently we have isolated further adenoviruses from an epidemic en masse among fatted lambs.

The epidemic occurred in the end of January among fatted lambs of a large farm. The cross-breed lambs, weighing 12—13 kgs, were transported from different farms and collected in five modern buildings, 2000 animals in each. The lambs were given the same premixed food already 2—3 weeks before transportation. Seven—eight days after arrival the onset of diarrhoea, 2—3 days later sneezing, serous nasal discharge and lacrimation could be observed. Sudden deaths also occurred (approx. 10 per cent of the total losses), in these cases focal catarrhal pneumonia, catarrh of the upper respiratory tract, enteritis, enlarged peribronchial and intestinal lymph nodes were found pathologically. Histopathological examinations revealed acute alterations of inflammation and lymphoid hyperplasia in the lungs and in the lymph nodes. The diarrhoea ceased after approx. one week, but the acute respiratory symptoms persisted for a further week. The application of antibiotics did not result sufficiently. In the majority of the cases the respiratory disease became chronic. It was most likely due to the insufficient ventillation, high ammonium concentration and humidity. Cough could be observed for a long time, chronic bronchitis, bronchiolitis and peribronchial pneumonia were found. Both acute and chronic respiratory processes were often accompanied by secondary bacterial complications. Severe respiratory symptoms were seen in these cases with frequent deaths (90 per cent of total losses). *Pasteurella haemolytica* could be cultivated from the lungs of animals died in this period. From the beginning

of the epidemic 259 animals died and 430 were emergency slaughtered within two months. It is worth to note that altogether 378 lambs died and 1323 were emergency slaughtered during a whole year in 1973.

Viruses were isolated in embryonic sheep kidney cultures from a high number of freshly diseased lambs. The agents, isolated from the nasal and rectal swabs, proved to belong to the adenovirus group on the basis of their physicochemical and biological properties. In the convalescent sera 1 : 32—1 : 128 titre neutralizing antibodies were found against the isolated adenovirus. No antibodies were detected in the sera of freshly diseased lambs. Detailed histopathological and virological studies will be reported elsewhere.

## Summary

An epidemic en masse among fatted lambs is reported. The animals showed respiratory and intestinal symptoms, by necropsy catarrhal pneumonia, catarrh of the upper respiratory tract and enteritis were found. In majority of the cases the respiratory symptoms became chronic. Both acute and chronic processes were followed by bacterial complications. From the freshly diseased animals adenoviruses were isolated, the isolates played an aetiological role in the disease.

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Address of the authors: Dr. Sándor Belák, Dr. Vilmos Pálfi, 1143 Budapest, Hungária krt. 23, Hungary

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# SOME GROWTH CHARACTERISTICS OF GOOSE PARVOVIRUS STRAIN "B"

By

J. KISARY

Veterinary Medical Research Institute (Director: D. DERZSY),
Hungarian Academy of Sciences, Budapest

(Received June 5, 1974)

The causal agent of a fatal disease of goslings has been recently identified as a parvovirus (SCHETTLER, 1973; DANNACHER et al., 1974; KISARY and DERZSY, 1974). Amid characterization of the "B" virus strain many difficulties were encountered as far as the in vitro propagation was concerned. The "B" strain could be adapted to the cell culture system only when it was inoculated into the suspension of cells to be plated. Consequently, the goose parvovirus strain "B" needs rapidly dividing cells for replication as do other members of this virus group (BACHMANN, 1972; PARKER et al., 1970; SIEGL and GAUTSCHI, 1973).

The purpose of this work was to study the factors influencing the in vitro replication of the goose parvovirus strain "B" in order to define the optimal conditions for virus isolation and further studies of the virus.

## Materials and Methods

*Tissue culture and virus.* Goose embryo fibroblast cultures were made as has been earlier described (KISARY and DERZSY, 1974).

Goose parvovirus strain "B" in the 31st tissue-culture-passage was used.

*One-step growth experiment.* $6 \times 10^7$ freshly trypsinized cells were infected with $2 \times 10^5 TCID_{50}$ virus. After an adsorbing period of 30 min at 37°C in a $CO_2$ incubator the cells were centrifuged at low speed and washed with PBS three times. After the third centrifugation the cells were resuspended in growth medium and seeded into 20 plastic Petri dishes (60 mm). The cultures were incubated in a $CO_2$ incubator for 120 hours and samples were taken in every 12th hours. The growth medium was assayed for infectivity of extracellular virus and the three times washed and frozen-thawed cells for cell-associated virus. The samples were kept at —20°C until centrifuged before assayed.

*Post-seeding susceptibility of cells to the virus infection.* $6 \times 10^6$ freshly trypsinized cells were infected with $2 \times 100 TCID_{50}$ virus and incubated for 30 min at 37°C. After that, the cells were washed and centrifuged three times with PBS, finally they were resuspended in 10 ml growth medium and seeded into 2 plastic Petri dishes. Furthermore, 2—2 cultures were infected with $100 TCID_{50}$ virus at different intervals after the seeding.

The cultures were incubated for 5 days, respectively. Then they were frozen and thawed three times and centrifuged. The supernatants were kept at —20 °C until assayed for infectivity.

*The effect of cell concentration on the virus yield.* $5{,}5 \times 10^7$ cells were infected with $10^6 TCID_{50}$ virus. After the adsorption period of 30 min at 37 °C the cells were washed and centrifuged three times at low speed. Finally the resuspended cells were seeded into 10 plastic Petri dishes (60 mm) with increasing number ranging between $1—10 \times 10^6$ cells/dishes. After an incubation period of 5 days the cultures were assayed for infectivity.

## Results

*One-step growth curve.* The results of the one-step growth experiment are summarized by Fig. 1. Since the goose parvovirus strain "B" is very stable, residual infectivity could be detected at each sampling time before appearance of the progeny virus. It was detected first intracellularly at the 36th hour p.i. and at the 48th hour, extracellularly. The infective titre of the extracellular virus exceeded that of the intracellular one between 84—96 hours p.i. At the end of the growth cycle only 10% of the progeny virus remained cell-associated.

*Post-seeding susceptibility of cells to the virus infection.* The highest infective titre could be achieved when the virus was inoculated into the suspen-

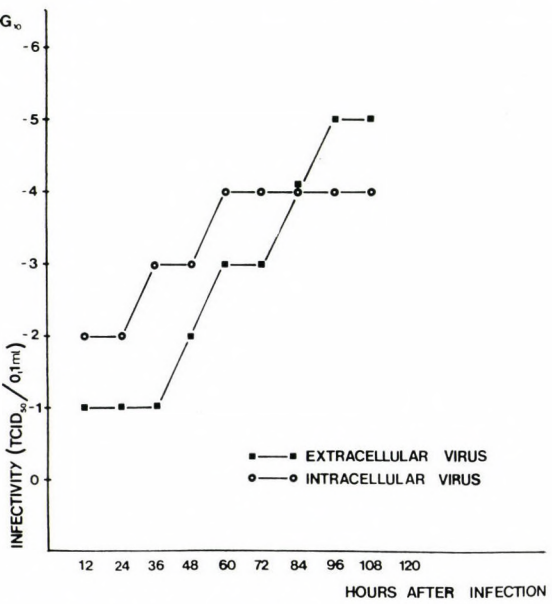


*Fig. 1.* One-step growth cycle of the goose parvovirus strain "B"

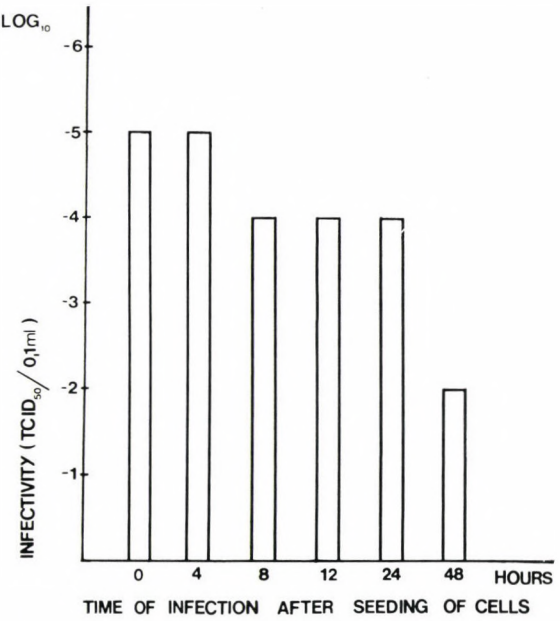


*Fig. 2.* Post-seeding susceptibility of cells to the virus infection

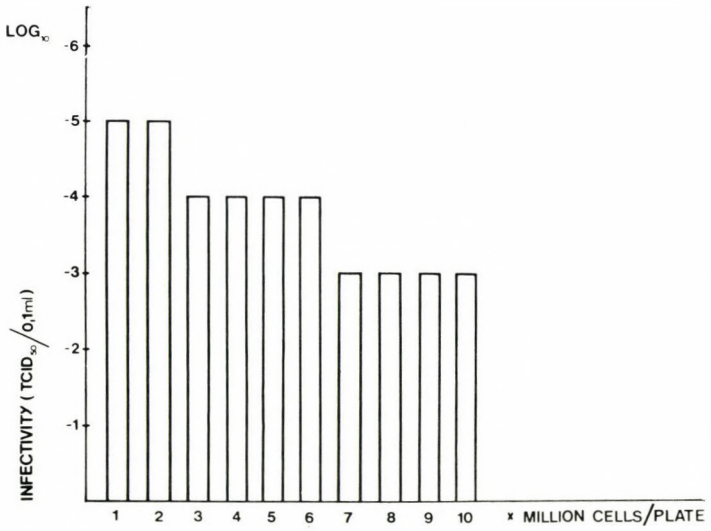


*Fig. 3.* The effect of the cell concentration on the virus yield

sion of cells to be plated or at the 4th hour after the seeding. The yield of the virus was lower by 1 log when cultures were infected between 4—24 hours postseeding. A striking decrease in the infectivity was found in case of cultures infected at 48th hour after the seeding of cells (Fig. 2).

*The effect of the cell concentration on the virus yield.* As it is shown in Fig. 3 the higher the concentration of cells was the lower infective titre could be obtained.

## Discussion

Earlier many attempts were made without success to adapt the egg-propagated goose parvovirus strain "B" to tissue culture system. However, when the virus was inoculated into the suspension of cells to be plated no further difficulties were encountered with its in vitro replication (KISARY and DERZSY, 1974).

In order to improve the methods used in work with this virus it seemed necessary to study its production kinetics and the factors influencing the in vitro replication.

The intracellular progeny virus could be detected after an eclipse period of 36 hours, while the extracellular one was first found after 48 hours p.i. Up to the 84th hour the infective titre of the intracellular virus was higher than that of the extracellular one. After 96 hours p.i. only the 10% of progeny viruses remained cell-associated.

SIEGL and GAUTSCHI (1973) studied the optimal conditions of parvovirus LuIII replication in synchronized cell cultures. They had come to the conclusion that the events of the viral nucleic acid synthesis were closely bound to the late S phase of cell cycle when the cells were in the status of "physiologic competence".

The goose parvovirus strain "B" also seemed to require growing cells for the replication. This was revealed in the slight — if any — virus replication when a completely confluent monolayer was infected, whereas, a relative high virus yield was obtained by inoculating the virus into the suspension of cells to be plated. On the other hand, the high concentration of cells to be seeded influenced unfavourably the conditions of virus replication by decreasing the number of the dividing cells.

On the basis of the results discussed above the highest yield of the goose parvovirus can be expected in tissue culture system if the virus is inoculated into the suspension of freshly trypsinized cells adjusted to the proper concentration.

## Summary

A study was performed on the replicative cycle of goose parvovirus strain "B" and factors influencing the virus replication in tissue culture system. The intracellular progeny virus was first detected after an eclipse period of 36 hours p.i. followed by the release of the extracellular progeny virus at the 48th hour p.i. The total cycle of the in vitro replication took about 96 hours when the 10% of the progeny viruses remained cell-associated. The post-seeding susceptibility of cells to the virus infection was demonstrated gradually decreasing. The high concentration of cells to be plated influenced unfavourably the virus yield.

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Address of the author: Dr. János KISARY, 1143 Budapest, Hungária krt 23, Hungary

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# ON THE SUSCEPTIBILITY OF THE DOMESTIC PIG TO THE HONG KONG SUBTYPE OF HUMAN INFLUENZA A VIRUS

By

J. ROMVÁRY, L. STIPKOVITS, Mária STIRLING-MÓCSY and E. FARKAS

Veterinary Medical Research Institute (Director: D. DERZSY) of the Hungarian Academy of Sciences, Budapest, Central Veterinary Institute (Director: A. VÁNYI), Budapest and Hungarian Society of Microbiology, Budapest

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Soon after the pandemic caused by the H2N2 subtype of the human influenza A virus in 1957, ROMVÁRY et al., (1962) isolated, from pigs kept in a fattening plant in Hungary, a strain sharing the human H2 haemagglutinin. This was the only strain with human H2 haemagglutinin isolated from pigs during the H2N2 era of the human influenza. In the same stock haemagglutination-inhibiting (HI) antibodies to the influenza A/Singapore/1/57 (H2N2) strain were demonstrated in 45% of the serum samples tested.

Early in 1969 during the so-called Hong Kong (H3N2) influenza pandemic ROMVÁRY and VIZY (1971) collected serum samples from pig stocks and succeeded in demonstrating anti-H3 antibodies in 13 to 73% of the sera. This was the first demonstration of Hong Kong influenza virus infection in pigs. Prompted by this finding, the World Health Organization (Geneva) organized a co-operative study on this subject. It was shown that in years 1970 and 1971 the Hong Kong virus was widespread in pigs, in some geographical areas even in higher percentage than found by us in Hungary. Late in 1971 ROMVÁRY isolated in India (State Farm, Haringhata-Kalyani, West Bengal) a haemagglutinating virus strain from tracheal and bronchial mucosa of an emergency-slaughtered pig 70 kg in body weight. The pig showed elevated temperature (40.0—40.8°C), serous nasal discharge, lacrimation, weariness and anorexia. Early in 1972 serum samples from 187 pigs, of those slaughtered in the abattoir nearest the above-named fattening plant, were tested for HI antibodies using the isolate as antigen; 113 pigs (60%) had antibodies up to a titre of between 1 : 16 and 1 : 512. Nearly the same titres were obtained with an antigen prepared from the influenza A/Hong Kong/1/68 (H3N2) strain. (The strain was kindly supplied by the World Influenza Centre, London.) With the isolate guinea pigs were immunized. The resulting antiserum inhibited the Hong Kong virus up to 1 : 256, indicating that the isolate was closely related to the reference strain.

## Results

Early in 1973, blood samples were collected from 861 pigs, mainly young sows in 42 farms situated in 13 counties of Hungary. In identical units of 13 of the stocks, sampling was repeated 20, 45 and 58 days later. Of the samples 471 (55%) proved to be positive for HI antibodies when tested against a strain influenza A/Hungary/1/73 (H3N2) from man in the same epidemic period when the blood samples were collected by us.

In each of the 13 counties 33 to 73% of the tested sera proved to be positive up to a dilution of 1 : 16 to 1 : 128. In some of the stocks the first screening failed to reveal positive sera, whereas 20, 45 and 58 days later antibodies could be demonstrated in 52, 67 and 92% of the tested animals (Table I).

In farm No. VI, on the other hand, 78% of the pigs were positive as early as on 20 January and this percentage did not change during the subsequent 45-day period.

In farm No. VII samples were collected from several units over a nearly three-month period. The seropositivity rate showed a well-defined rise.

**Table I**

Serial screenings in swine stocks for anti-Hong Kong HI antibodies

| Farm No. | Dates of screenings | No. of blood samples tested | Of these positive | |
|---|---|---|---|---|
| | | | No. | per cent |
| I | 18/1/73 | 20 | 0 | 0 |
| | 28/2/73 | 12 | 2 | 17 |
| II | 12/12/72 | 17 | 0 | 0 |
| | 8/2/73 | 12 | 11 | 91 |
| III | 18/1/73 | 18 | 0 | 0 |
| | 7/2/73 | 25 | 13 | 52 |
| IV | 4/1/73 | 15 | 0 | 0 |
| | 10/3/73 | 18 | 12 | 67 |
| V* | 24/1/73 | 19 | 1 | 5 |
| | 23/2/73 | 13 | 10 | 77 |
| VI* | 20/1/73 | 18 | 14 | 78 |
| | 22/2/73 | 26 | 20 | 77 |
| | 6/3/73 | 22 | 18 | 82 |
| VII | 13/12/72 | 15 | 5 | 33 |
| | 7/2/73 | 20 | 9 | 45 |
| | 15—17/2/73 | 37 | 24 | 65 |
| | 3/3/73 | 23 | 20 | 87 |

* = Farm No. V is 25 km away from farm No. VI

In several stocks which showed a positivity over 60% in the first two months of 1973, the surviving sows and boars of about one year of age had antibodies in 8 to 17%. At the same time the seropositivity rate for the animals aged several years was found at 28%.

In farm *H.* clinical symptoms and postmortem findings proved the prevalence of enzootic bronchopneumonia for a long period of time. Piglets over one month of age, weanlings, and also piglets kept in the fattening plant, were affected. The resulting loss had approached 25% by the weanling age. Some sows showed the mastitis-metritis syndrome. In some case *Mycoplasma hyopneumoniae* was isolated from the lungs, whereas in one of these the nasal and laryngeal mucosa yielded a haemagglutinating virus strain which proved to belong to the Hong Kong subtype.

*Attempts to infect pigs with Hong Kong virus strains*

Minnesota and conventional piglets 8 to 10 weeks of age were inoculated intranasally with the strain Hungary/1/73 of human origin. The majority of the inoculated piglets and their litter-mates developed fever up to 40.3—40.9°C 3 to 5 days after inoculation. Blood was taken from the infected piglets on the 26th postinoculation day and the sera were tested for HI antibodies, using pigeon erythrocytes and an antigen prepared from the strain 1/73 by ether extraction. The antibody titres of the infected miniature piglets reached 1 : 64 or 1 : 128, while those of the litter-mate control sera did not exceed 1 : 16. The virus-infected conventional piglets became accidentally infected by *Escherichia coli* strain of haemolytic activity. The postinfection serum titres of these piglets did not exceed the 1 : 16 level. The virus could be re-isolated from the artificially infected animals, even from those infected by the haemolytic *E. coli*, and also from the contact controls, between the 2nd and 7th postinfection days.

With the 2nd passage of our Hong Kong subtype strain of piglet origin three miniature piglets were inoculated. These and all the three contact controls developed fever. The virus could be re-isolated from inoculated piglets from the 24th hour on through a week and from the controls between postinoculation days 2 and 7. Three weeks after inoculation the HI titre attained 1 : 256—1 : 512 in the artificially infected piglets and 1 : 32—1 : 128 in the contacts. Seven days after a booster dose from the same strain the titres were found to be higher by two or three $\log_2$ units.

In a further experiment one piglet of a three-month-old Minnesota litter was infected with our Hong Kong subtype strain of piglet origin. The littermates were isolated from the infected litter for varying number of days, then placed back. The virus was recovered from those placed back 4 or 9 days postinoculation up to the 6th day of exposure, but could not be recovered from

those placed back 19 days or more after artificial inoculation. On the 51st day, *i.e.* two weeks after the last (*i.e.*, 6th) litter-mate had been exposed, homologous antibodies were demonstrated up to 1 : 16 or 1 : 32 even in the sera of those contacts which had not yielded virus. This means that the virus persisted for at least 37 days in the litter. By the 8th day after a booster dose the titres had risen by 2 or 3 $\log_2$ units.

## Discussion

In four counties, all of which were already afflicted by the influenza outbreak as early as in the second half of December, 1972, 60—100% of the collected serum samples proved to be positive for HI antibodies when tested with the virus simultaneously prevalent in humans. Some relationship between the time of appearance of the outbreak in humans and the antibody response of pigs was also observed. However, due to the low number of samples collected from some counties, the geographical spreading of the virus in pig stocks cannot be followed on the basis of the present studies. Anyhow, considering the rapidly rising rate of seropositivity, it seems to be likely that the virus spread rapidly from pig to pig, showing a high contagiosity. The primary source of infection must have been personnel of farm.

In the second half of January, the seropositivity rate was 5% in farm No. V as contrasted to the 78% for farm No. VI. In the following 30-day period the seropositivity for farm No. V reached the latter level. It should be noted that the human population of the village to which farm No. V belongs was reached by the intensive wave of the epidemic some three weeks after the first blood sampling.

The anti-Hong Kong antibodies had disappeared or fallen to a rather low level by the 6th to 10th postepidemic month. Somewhat higher titres persisted in the sera of older sows and boars, supposedly because the antigenic stimulus was a secondary one for some of these.

The virus strain sharing the human H3 haemagglutinin was isolated by us from a suckling piglet simultaneously infected by *M.hyopneumoniae*. The double infection and the consequent decrease in milk secretion must have played a role in the 25% loss of suckling piglets. In the respect it seems to be of interest that the pigs which were accidentally infected by an *E. coli* strain of haemolytic activity gave poor HI antibody response to the influenza virus infection.

The persistence of the Hong Kong virus in our experiment for at least 37 days suggests that an influenza outbreak in pigs may last as long as there are nonimmune animals in the stock. Earlier, considering that the virus usually cannot be re-isolated after the first week (Tateno et al., 1965; Francis and Maassab, 1965; Hoyle, 1968), the virus was believed to disappear very

soon. Recently WILKINSON and BORLAND (1972) observed multiplication of the A/PR8/34/HON1 virus in human embryonic lung monolayer cultures as late as on the 52nd day after infection, suggesting that virus-producing cells may long survive.

## Summary

Rapid spreading of influenza virus infection was observed in swine stocks during an influenza outbreak caused by a derivative of the Hong Kong virus. A considerable part of the tested stocks became affected within 20 to 58 days. At the end of this period 52% to 92% of the animals in the different stocks had haemagglutination-inhibiting antibodies to an influenza virus strain isolated from the simultaneous outbreak from man. Antibodies remained demonstrable for 6 to 10 months. It was shown in an experimental model that closed stocks may remain infected for relatively long periods of time, actually 37 days.

A virus strain sharing the H3 haemagglutinin of human influenza A virus was isolated from the nasotracheal mucosa of a diseased one-month-old piglet. In the same affected stock, where loss of suckling piglets had reached 25%, *Mycoplasma hyopneumoniae* was isolated from the lungs of piglets. The double infection associated with a reduced milk secretion may be responsible for such losses.

In piglets the simultaneous presence of haemolytic *E. coli* and Hong Kong influenza infection prevents the producing of specific HI-antibodies to the latter causative agent.

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Address of the authors: Dr. József ROMVÁRY, Dr. László STIPKOVITS, 1143 Budapest, Hungária krt. 23; Dr. Mária STIRLING-MÓCSY, 1104 Budapest, Sörgyár u. 41—43; Dr. Elek FARKAS, 1124 Budapest, Zsámbéki u. 15, Hungary

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# ISOLATION OF INFLUENZA A VIRUS STRAINS FROM CATTLE

## PRELIMINARY REPORT

By

J. TANYI, J. ROMVÁRY, P. ÁLDÁSY and Zsuzsa MÁTHÉ

Veterinary Institute (Director: R. HENGEL), Debrecen, Department of Animal Physiology and Animal Health, Agricultural University (Director: F. MUNKÁCSI), Debrecen, Veterinary Medical Research Institute (Director: D. DERZSY), Hungarian Academy of Sciences, Budapest and Veterinary Institute (Director: P. ÁLDÁSY), Miskolc

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The occurrence of influenza viruses in animals is in the focus of interest all over the world. Investigations in this field are aimed at revealing (i) the aetiological role of these viruses in animal diseases, (ii) the susceptibility of animals to human influenza viruses, (iii) the changes in the antigens of the influenza virus envelope and (iv) the mechanism of the persistence of influenza viruses during interepidemic periods. Influenza A viruses have been isolated from outbreaks affecting horses, pigs and birds.

In Hungary, influenza A virus strains bearing the human H2 haemagglutinin were isolated in 1959—1960 from pig and sheep as reported by ROMVÁRY et al. Afterwards virus strains sharing the H3 haemagglutinin of human influenza A virus were isolated by ROMVÁRY from one-month-old piglet, turtle dove and dogs, respectively. In 1970—1971 Soviet authors (IZMAILOV et al., 1973; FATKHUDDINOVA et al., 1973; WHO Newsletter, 1973) isolated influenza virus strains from sporadic cases, and during an outbreak, of respiratory disease in cattle. These strains appeared to be closely related to the prototype strain of the H3N2 subtype of influenza A virus (strain A/Hong Kong/1/68) as confirmed by G. C. SCHLID (World Influenza Centre, London). All these isolations took place in a period when the human population was afflicted by an outbreak caused by a closely related virus.

The present paper is a preliminary report on our recent investigations which have been resulted in isolating three strains of infuenza A virus from diseased cattle.

### Materials and Methods

Nasal swabs of acutely ill animals and samples from the borders of intact and acute catarrhal pulmonary areas of emergency-slaughtered animals were examined. The samples were homogenized, and antibiotics were added. Each sample was inoculated into the amnioallantoic cavity of 5 embryonated hen's eggs and 5 embryonated duck eggs preincubated for 9 to 12 and 10 to 15 days,

respectively. The eggs were opened after 3 to 7 days reincubation or as the embryo has died. In several cases four blind passages were performed.

In the haemagglutination and haemagglutination inhibition (HI) tests chicken and guinea pig erythrocytes were applied. The serum samples were pretreated with heat, trypsin and potassium periodate as directed by WHO circular in 1967. Immunodiffusion test was performed as described by BEARD (1970).

## Results

*Cases.* Virus was isolated from three stocks each consisting of 50 to 200 cattle including calves 4 to 6 months of age, young cattle and beef cattle. The animals were suffering from the so-called "epizootic cough" associated with general (anorexia, lassitude, *etc.*) and respiratory symptoms (nasal discharge and lacrimation; deep, initially dry, then wet, strong coughing). The outbreak ceased in a stock, depending on the size of the stock, in 1 to 3 weeks. Several animals were lost. Postmortem examination showed small areas with acute catarrhal pneumonia as well as alveolar and interstitial emphysema and bronchitis, occasionally with asphyxial haemorrhages. No gross changes were seen in other organs. Bacteria could not be isolated.

*Virological investigations.* Virus was isolated in one case without blind passage and in two cases in the second or third passage in embryonated hen's eggs. Several embryos died on the 4th day, the remaining eggs were opened 3 to 7 days after inoculation. The pretreated positive amnioallantoic fluids formed a precipitation bend with anti-influenza human and bird sera.

In the HI test no inhibition was obtained with antisera to the reference strains of human, equine, swine and avian influenza A viruses. The set of avian influenza viruses was kindly supplied by the World Influenza Centre, London. One of the strains was submitted to the World Influenza Centre, London, for identification. It was found by G. C. SCHILD that its neuraminidase antigen is identical with the N2 antigen of human influenza A virus and its haemagglutinin is unrelated to the influenza A virus strains Hong Kong/1/68, Singapore/1/57, FMI/47, PR8/34 and the fowl plague virus. Postepidemic sera were obtained from two of the three stocks yielding virus, 3 to 5 weeks after the outbreak, 10 from one stock and 20 from the other. All the HI titres to the homologous virus ranged between 1 : 10 and 1 : 80. In the surroundings of the farms yielding virus no human influenza outbreak was noticed in the months preceding the outbreaks in animals.

## Conclusions

The results presented here suggest that, besides parainfluenza-3 virus (REISINGER et. al., 1959), adenoviruses (KLEIN et al., 1959, and others), rhinovirus (BÖGEL and BÖHM, 1962) and other viruses, influenza A virus strains may play an aetiological role in "epizootic cough" of cattle. Although the outbreak spread rapidly, the course of the cases yielding virus was relatively slow.

Further investigations are needed to clarify the aetiological role of the influenza A virus in "epizootic cough". From the aspect of public health it is of interest that one of the virus strains shares the N2 neuraminidase with the influenza A virus strains having been prevalent in humans since 1957. It should be noted that our isolate is different from the strains isolated from cattle in the Soviet Union.

## Summary

Three influenza A virus strains were isolated from cattle stocks consisting of calves, young cattle and beef cattle suffering from a disease diagnosed as "epizootic cough". One of the isolates has been proved different from all the strains isolated from man, horses, swine and birds so far, in bearing a human influenza neuraminidase antigen (N2) and an unknown haemagglutinin.

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Address of the authors: Dr. János TANYI, 4031. Debrecen Széchenyi út 94; Dr. József ROMVÁRY, 1143 Budapest, Hungária krt. 23; Dr. Pál ÁLDÁSY, Dr. Zsuzsa MÁTHÉ, 3525 Miskolc, Dóczy u. 6, Hungary.

# VARIA

The international Committee on Laboratory Animals (ICLA) will hold its 6th Symposium in Thessaloniki, Greece, on 8—12 July 1975 on the themes:

1. Laboratory Animals in the study of reproduction.
2. Training in laboratory animal science.

Interested scientists are invited to submit papers related to these main topics. Two hundred words abstracts should be mailed before February 1st, 1975 to the Chairman of the Programme Committee:

Dr. Nicolae Simionescu
Yale University School of Medicine
Cell Biology Department
333, Cedar Street
New Haven, Conn. 06510
U.S.A.

Proceedings of the Symposium will be published.
Further details are available from the Chairman of the Programme Committee.

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ACTA VETERINARIA

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# РЕЗЮМЕ

## МИГРИРУЮЩАЯ ЛИЧИНКА NEOASCARIS VITULORUM В ЛАБОРАТОРНЫХ ЖИВОТНЫХ

О. А. АТАЛЛАХ, И. МОССАЛАМ и М. С. АБДЕЛ-РАХМАН

Лабораторные животные и цыплята заражались яйцами **Neoascaris vitulorum**, содержащими инвазионные личинки.

Личинки обнаруживались в печени, легких, сердце, почках и лимфоузлах инвазированных животных. Изменения появлялись, как правило, в виде гранулем и наиболее тяжелыми были в морской свинке. Авторы считают, что личинки **Neoascaris vitulorum** могут инвазировать и человека.

## НАУЧНО-ИССЛЕДОВАТЕЛЬСКАЯ РАБОТА ПО КОКЦИДИЯМ И АПИКОМПЛЕКСАМ

Б. МЮЛЕР и Э. ШОЛТИСЕК

Ультраструктура отдельных представителей подтипа **Apicomplexa (Gregarinida Coccidia, Haemosporidia, Piroplasmida)** группой специалистов в Бонне при сотрудничестве американских, французских, австрийских и венгерских специалистов изучается дольше 15 лет. Благодаря этой работе обнаружено, что внутриклеточные формы токсоплазм и мерозоиты некоторых аймерий по своей структуре очень сходны и выявлены детали тонкого строения других паразитических групп. При помощи электронной микроскопии прослежены сексуальное и асексуальное развитие и в этом отношении между разными группами обнаружено или сходство, или отличие. Их исследования по взаимоотношению хозяин-паразит раскрыли тоже немало нового и дополнили данные цитохимических исследований проводившихся на других местах.

Задачей дальнейших электронно-микроскопических исследований является, между прочим, лучшее изучение ультраструктуры гамонтов, раскрытие образования флагелярного комплекса и базальных телец; кроме этого существенным является расшифровка внутриклеточного метаболического и энзимного механизмов, то есть изучение вредного действия паразитизма на организм хозяина.

## ЗАРАЗНЫЙ (ВИРУСНЫЙ) ЭНТЕРИТ, НОВОЕ ЗАБОЛЕВАНИЕ ГУСЯТ В СССР

Л. М. КОНТРИМАВИЧУС

Докладывается о результатах начавшихся в 1963 году исследований по заразному (вирусному) энтериту гусят, раньше неизвестному заболеванию в СССР. Заразным началом является вирус, ресистентный к хлороформу и температуре, который размножается в гусином зародыше; после нескольких пассажей он убивает зародыш и показатель $LD_{50}$ доходит до $10^{-4}$—$10^{-6}$. Вирус неидентичен с вирусом чумы птиц и вирусами утиного гепатита, хотя последние патогенны для гусят. Данный вирус распространяется при посредстве яиц и в некоторых случаях может быть причиной гибели 80—100% животных. Эпизоотология, клиника, вызываемые вирусом патологоанатомические изменения не отличаются от изменений, описанных в разных странах при разном наименовании того же этиологического фактора.

## ЭКСПЕРИМЕНТАЛЬНОЕ ЗАРАЖЕНИЕ ЯГНЯТ РЕОВИРУСОМ ТИПА 1, ИЗОЛИРОВАННЫМ ИЗ ВЗРОСЛЫХ ОВЕЦ

Ш. БЕЛАК и В. ПАЛФИ

Ягнята экспериментально заражались реовирусом типа 1 (H/II), изолированным из взрослых овец при естественной вспышке заразы. У 2—4-недельных зараженных в носовую полость и трахею ягнят после 4—6-дневной инкубации температура повысилась на 0,6—0,9 °С, наступило слезотечение, истечение слизи из носа, чихание, одышка и понос. У ягнят, убитых к 8-у дню после заражения, наблюдались энтерит, катар верхних дыхательных путей, диффузная интерстициальная пневмония. У одного ягненка со слабой ресистентностью эти признаки обострились и он к 11-у дню после заражения погиб. При вскрытии обнаружены энтерит, катар верхних дыхательных путей и множественная очаговая катаральная пневмония. Клинические признаки болезни у выживших ягнят к концу 3-й недели утихомирились и в конвалесцентных сыворотках обнаруживались специфические нейтрализирующие антитела против штамма H/II в титре 1 : 64—1 : 128. Вирус удалось реизолировать из тампонатов носовой полости и прямой кишки к 4.—9. дням после заражения. На основании экспериментальных данных и наблюдений в практике авторы приходят к заключению, согласно которому реовирусы могут играть роль в этиологии респираторных и кишечных заболеваний молодых ягнят.

## ХАРАКТЕРИЗАЦИЯ ОДНОГО ВИРУСА ХЕРПЕС, ИЗОЛИРОВАННОГО ИЗ СПОНТАННО ДЕГЕНЕРИРОВАВШЕЙ БЫЧЬЕЙ ПОЧЕЧНОЙ КЛЕТОЧНОЙ КУЛЬТУРЫ

Ш. БЕЛАК и В. ПАЛФИ

Докладывается об изолировании одного вируса из спонтанно дегенерировавшей бычьей почечной культуры. Изолят на основании физико-химических, морфологических и биологических свойств представляет собой один вирус группы херпес. Серологически он близок бычьему херпесному вирусному штамму **Móvár 33/65**. В экспериментах искусственного заражения вирусный штамм оказался непатогенным. Факт изоляции является новой информацией того, что здоровые телята могут быть носителями вирусов группы херпес.

## ЭЛЕКТРОННО-МИКРОСКОПИЧЕСКОЕ ИЗУЧЕНИЕ РАННИХ ИЗМЕНЕНИЙ ПРИ БОЛЕЗНИ МАРЕКА

### I. ЦИТОМОРФОЛОГИЯ ИЗМЕНЕНИЙ

Х. Н. ТАКУР

При помощи электронного микроскопа изучены опухоли и изменения в цыплятах после искусственного заражения вирусом, изолированным при острой форме болезни Марека (**MD/7/1970**). Описывается цитоморфология опухолевой клетки лимфоидного характера. Опухолевые клетки нуждались в цитоплазматических клеточных органеллах и их ядра часто показывали характерные выпячивания и впячивания. Они обладали морфологией, характерной для опухолевых клеток.

## ЭЛЕКТРОННО-МИКРОСКОПИЧЕСКОЕ ИЗУЧЕНИЕ РАННИХ ИЗМЕНЕНИЙ ПРИ БОЛЕЗНИ МАРЕКА

### II. ГИСТОГЕНЕЗ ИЗМЕНЕНИЙ

Х. Н. ТАКУР

Данные изучения гистогенеза ранних изменений при болезни Марека подтвердили правильность наших наблюдений, полученных при помощи нормального микроскопа. Пролиферации были периваскулярного характера и исходили из стенок малых кровеносных сосудов, сначала в виде пролиферации мультипотентных — адвентициальных — клеток. Инфильтрация и пролиферация плеоморфных лимфоидных клеток ябляется характерным явлением образования опухолей при болезни Марека. Пролиферации в висцеральных органах и в периферических нервах не отличались друг от друга.

## ХАРАКТЕРИЗАЦИЯ ОДНОГО ОВЕЧЬЕГО ШТАММА ВИРУСА ПАРАИНФЛЮЭНЦЫ-3, ИЗОЛИРОВАННОГО ИЗ ОВЦЫ С СИМПТОМАМИ ЗАБОЛЕВАНИЯ ДЫХАТЕЛЬНЫХ ПУТЕЙ

Ш. БЕЛАК и В. ПАЛФИ

Вирусный штамм изолирован из тампонатов носовой полости 4-месячных мериносовых овец, показывающих слабые признаки заболеваемости дыхательных путей. Изолят, обозначенный НОВ/II, характеризовался физико-химическими и серологическими пробами. Данные характеризации показали, что вирус принадлежит к группе вирусов параинфлюэнца-3. Согласно серологическим исследованиям изолят можно считать одним среди этиологических факторов респираторной болезни овец. Согласно авторам не исключена возможность передачи вирусов параинфлюэнца-3 овцами крупному рогатому скоту.

## ВИРУСНОЕ ЗАБОЛЕВАНИЕ ГУСЯТ. IV. ХАРАКТЕРИЗАЦИЯ ПАТОГЕННОГО НАЧАЛА В ТКАНЕВЫХ КУЛЬТУРАХ

Я. КИШАРИ и Д. ДЕРЖИ

Вирусный штамм «В», изолированный из тушек погибших гусят, адаптировали к тканевой культуре фибробластов гусиного зародыша. Вирус вызывал образование эозинофильных включений в ядрах пораженных клеток. В физико-химических пробах вирус оказался устойчивым к хлороформу и температуре при pH 3. Пробой торможения 5-йодо-2-деоксиуридином обнаружено, что ядерная кислота является типа дезоксирибонуклеиновой кислоты. Отрицательно окрашенные вирионы при изучении электронным микроскопом были диаметра 20—22 миллимикронов. На основании этих свойств авторы приходят к убеждению, что вирусный штамм «В» нужно включить в группу парвовирусов птичьего происхождения.

## ИСТОЧНИК ИНВАЗИИ ПРИ ТОКСОПЛАЗМАТОЗЕ КЕНГУРУ ЗООПАРКОВ

М. ДОБОШ-КОВАЧ, Я. МЕСАРОШ, Л. ПЕЛЛЕРДИ и А. БАЛШАИ

Авторами диагносцирован генерализированный токсоплазматоз у трех совместно содержавшихся кенгуру (**Thylogale eugenii**) будапештского зоопарка. Диагноз инвазии поставлен на основании патогистологической картины и путем искусственного заражения. Экспериментальным животным использована изолированно содержавшаяся молодая кошка, кормившаяся органами погибших кенгуру. После инвазирования кошка начала выделять ооцисты **T. gondii**, которыми впоследствии удалось после их споруляции инвазировать мышей.

Согласно данным наблюдений кенгуру инвазировались кормом, загрязенным испражнениями кошки. В кошачьих испражнениях, в корме и свежих испражнениях кошки, посещавшей помещение с кормом для кенгуру, обнаружены ооцисты токсоплазм, которыми тоже удалось инвазировать лабораторных мышей. Указывается на роль гигиены и значение кошки в распространении токсоплазматоза.

## МУКОИДНЫЙ ЭНТЕРИТ У КРОЛИКА, СВЯЗАННЫЙ С ВЫЗВАННЫМИ E. coli ИЗМЕНЕНИЯМИ УГЛЕВОДНОГО ОБМЕНА

Ф. ВЕТЕШИ и Ф. КУТАШ

Биохимическими, гистохимическими и электронно-микроскопическими методами изучалось действие естественного мукоидного энтерита на углеводный обмен у кролика. Изучены не только больные, но и здоровые кролики того же поголовья.

Углеводный обмен кроликов, страдающих мукоидным энтеритом, характерен следующими признаками: усиление гликогенолиза в печени и сердечной мышце, высокая активность печеночной и почечной глюкоза-6-фосфатаз, агликозурическая гипергликемия, накопление гликогена в проксимальных канальцах почек.

Авторы считают, что тяжелые перебои углеводного обмена — в первую очень изнурение клеточного гликогенного депо — являются причиной летального исхода болезни.

## ИЗУЧЕНИЕ ПАТОГЕННОСТИ ТОКСОПЛАЗМ ДЛЯ МЫШЕЙ

Л. ПЕЛЛЕРДИ и М. ДОБОШ-КОВАЧ

Токсоплазматозный штамм «Будапешт» изолирован из неизвестного происхождения кошки таким образом, что выделяющимися ооцистами после их споруляции через рот инвазировались мыши. Штамм содержался путем пероральной реинвазии с промежутками времени в 15—187 дней цистами, содержащимися в мышином мозге. Патогенность штамма для мышей сравнивалась с таковой штамма «Гаил» из Бонна (ФРГ). Между упомянутыми штаммами в этом отношении разницы не обнаружено. После инвазии ооцистами из кошки большинство мышей к 5—11-у дню погибло даже в том случае, если одна мышь получила только 5 ооцист.

При вскрытии павших мышей наблюдалось расширение просвета тонкого отдела кишечника, накопление в нем жидкости, эдематозное набухание стенки кишечника, перебои периферического кровеобращения, увеличение лимфатических пляшек кишок и брыжеечных лимфоузлов, размножение колибациллов в кишке. В разных органах (слизистая кишечника, лимфатические пляшки, брыжеечные лимфоузлы, редко печень) гистологически обнаружены стадии развития токсоплазм.

Паталогоанатомические и патогистологические изменения указывают на то, что мыши погибли из-за острого шока. На основании результата бактериологического изучения кишечного содержимого можно заключить, что в гибели мышей, инвазированных ооцистами токсоплазм, важную роль играет консеквентное размножение колибациллов, то есть шок от колибациллезной энтеротоксемии.

## ПНЕЙМОЭНТЕРИТ СРЕДИ ЯГНЯТ, ВЫЗВАННЫЙ АДЕНОВИРУСОМ. ПРЕДВАРИТЕЛЬНОЕ СООБЩЕНИЕ

Ш. БЕЛАК и В. ПАЛФИ

Докладывается о массовой эпизоотии среди откормочных ягнят. Животные показывали респираторные и кишечные симптомы. При вскрытии обнаружены катаральная пневмония, катар верхних дыхательных путей и энтерит. В большинстве случаев респираторные признаки были хроническими. Как острую, так и хроническую форму болезни сопровождали бактериологические компликации. Из свежих тушек павших животных изолированы аденовирусы. Изоляты играли роль этиологического фактора в наступлении болезни.

## НЕКОТОРЫЕ ХАРАКТЕРНЫЕ ЧЕРТЫ РОСТА ШТАММА «В» ГУСИНОГО ПАРВОВИРУСА

Я. КИШАРИ

Изучалась кривая одноступенчатого развития и факторы, действующие на репликацию в тканевой культуре штамма «В» гусиного парвовируса. Внутриклеточные и внеклеточные наследственные вибрионы обнаруживались 24—36 и 36—48 часов после заражения соответственно. Полный цикл репликации in vitro равнялся примерно 96 часам, к концу этого срока 10% заново образовавшихся вибрионов осталось связано с клетками. Чувствительность клеток к вирусу после высева постепенно понижалась. Высокая концентрация клеток при высеве отрицательно действует на количество получаемого вируса.

## ВОСПРИИМЧИВОСТЬ СВИНЬИ К ПОДТИПУ ГОНГ-КОНГ ВИРУСА ЧЕЛОВЕЧЬЕЙ ИНФЛЮЭНЦЫ А

Й. РОМВАРИ, Л. ШТИПКОВИЧ, М. ШТИРЛИНГ-МОЧИ и Э. ФАРКАШ

Во время эпидемии инфлюэнцы, вызванной производным вируса гонг-конг, наблюдалось быстрое распространение инфекции вирусом инфлюэнцы среди свиней. Значительная часть изученных поголовьев заразилась в течение 20—58 дней. К концу указанного срока времени 52—92% животных разных поголовьев обладало гемаглютинацию тормозящими антителами против одного вирусного штамма инфлюэнцы, изолированного при симультанных вспышках заразы среди людей. Антитела были выявимы в течение 6—10 месяцев. На экспериментальной модели показано, что замкнутые поголовья оставались зараженными относительно продолжительное время, фактически 37 дней.

Из слизистой носовой полости и трахеи одномесячного больного поросенка изолирован вирусный штамм, содержащий гемагглютинин «Н3» человечьего вируса инфлюэнцы А. В другом неблагополучном поголовье, где гибель среди поросят достигла 25%, из легких одного поросенка изолирована **Mycoplasma hyopneumoniae**. Причиной этой высокой смертности среди поросят явилась эта двойная инфекция и связанное с ней понижение продукции молока у свиноматок.

Симультанное наличие в поросятах гемолитических колибациллов и инфлюэнцы гонг-конг тормозило продукцию специфических гемагглютинацию тормозящих антител против последнего этиологического фактора.

## ИЗОЛЯЦИЯ ВИРУСНЫХ ШТАММОВ ИНФЛЮЭНЦЫ А ИЗ КРУПНОГО РОГАТОГО СКОТА

Я. ТАНИ, Й. РОМВАРИ, П. АЛДАШИ и Ж. МАТЭ

Три вирусных штамма инфлюэнцы А изолированы в поголовьях крупного рогатого скота, состоящих из телят, молодняка и взрослых животных. Животные страдали т. наз. «заразным кашлем». Один среди изолятов отличался от всех штаммов, изолированных по настоящее время из людей, лошадей, свиней и птиц, наличием антигена ($N_2$) человечьей инфлюэнцы и одного неизвестного гемагглютинина.

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# AN INFECTIOUS INFLAMMATORY DISEASE OF CLOACA AND PENIS IN GEESE (GOOSE GONORRHOEA)

## I. EPIZOOTOLOGICAL, CLINICAL, PATHOLOGICAL OBSERVATIONS AND CONTROL

By

I. Szép, Mária Pataky and Gy. Nagy

Department of Physiology and Animal Health (Head: I. Szép), University of Agricultural Sciences, Gödöllő

(Received July 10, 1972)

Large-scale goose keeping involves many veterinary and production problems. A major drawback of economic production is egg infertility, which causes great losses in many goose farms, but little is still known about its causes. During the last two years, increasing incidence of a previously unknown reproductive organ disease was observed in goose flocks in Hungary.

The traumatic prolapse of the penis of male ducks and geese has long been known by the veterinarians. This condition usually arises from the secondary bacterial infection of a mechanical injury inflicted during copulation, but it never spreads from bird to bird. As a rule not more than 0.5 to 1% of the drakes and ganders become affected during the reproductive period. In contrast to this the new venereal disease involves 70 to 80% of the male geese in the affected farms.

The scanty literary data available on venereal diseases of poultry were of little aid in identifying the new gander disease, because a mass incidence of cloacal inflammation was hitherto only reported among hens and female ducks.

Lührs (1955) described the symptoms of the chicken disease as loss of condition, reddening and swelling of the cloacal opening, pseudomembranaceous lesions and ulceration of the cloacal mucosa and reduction of egg yield. The illness lasted a few weeks and was usually fatal in outcome. The author obtained good therapeutic results with Hedan preparations, but failed to identify the causal agent and transmission experiments were also negative.

The infectious inflammation of the cloaca of ducks was reported by Wagener and Harms (1948), later by Quaglio (1959) and Svitavsky (1965). These authors are of the opinion that the male ducks of the flock become affected first. Swelling and prolapse of the penis are followed by the formation of thick, yellowish scabs on penis and cloacal mucosa. Duck layers often have scabs in the skin-mucosa transition zone of the cloacal region; the scabs dip 1—2 cm deep into the cloaca. Although the disease is generally regarded as infectious, the causal agent is still not known. Experimental infections failed. The outbreaks last several weeks in the flocks. Therapeutic attempts with sulphonamide preparations and adstringents were ineffective. In Hungary, Palya (1971) reported that in certain goose flocks where egg infertility was high, several ganders had a swollen, prolapsed penis, often with secondary inflammation of the mucosal part. The egg infertility was nevertheless attributed to feeding errors and damage by fungal toxins.

## Description of the gander disease

Egg infertility and a severe disease of the male organ of ganders were first reported to our Department from several goose farms in 1970. In January, 1971, at the beginning of the laying season, outbreaks of the same disease occurred in many goose flocks. The simultaneous incidence in various flocks kept at different levels of management technology *ab ovo* suggested an infectious aetiology, unrelated to management system and dietary factors.

The first symptom was a temporary loss of appetite, followed by inflammation of the cloaca and — in male birds — by reddening and necrotic inflammation of the penis which led in severe cases to prolapse and partial loss of the male organ. The birds were generally listless and 10% of the ganders died in the infected flocks within a few weeks. Egg infertility increased abruptly with the progression of the outbreak and the female birds stopped laying eggs within 4—5 weeks after the onset.

Diseased and dead birds submitted from different parts of the country showed the same symptoms and gross lesions, the only difference being the degree of the changes, depending on the early or advanced stage of the disease.

In the initial stage, the penis can be exposed by the massage procedure employed for artificial insemination, it shows swelling and mucosal reddening only nearby the ventral wall of the cloaca, and the scalloping of the spiral seminal duct is well preserved (Fig. 1). Later exposure of the penis encounters difficulty, the penis is coated by a foamy mucus and the cloacal mucosa exhibits swelling and a marked reddening (Fig. 2). After the acute stage, inflammation involves the entire male organ. The mucous membrane assumes a purple or violet-reddish shade, the seminal duct disappears, a yellowish, scab-like deposition (Fig. 3) and ulceration (Fig. 4) develop until, finally, the inflamed part becomes detached (Fig. 5). The cloacal region is swollen and hard. Prolapse and incarceration sometimes result in necrosis and detachment of the entire penis. According to newer observations, prolapse, demarcation at the root and detachment of the male organ can also occur without preceding severe inflammatory phenomena.

The main — often only — symptom shown by the layers is the swelling and hardness of the cloacal region. In addition pseudomembranaceous diphtheroid depositions are frequently found on the cloacal mucosa.

The course of the disease is very rapid, as shown in Fig. 6, which shows the curve for an outbreak in a large flock. The flock, 11,256 birds, was kept in a traditional covered yard of 2700 $m^2$ floor space and no water was available in the run. The outbreak set on after a latency for 5—6 days between 9 and 17 February 1972, following the beginning of mating. Egg production reached the maximal level very soon, then fell gradually and simultaneously the feed



*Fig. 1.* Normal male organ of gander. Note the patency and scalloping margins of the seminal duct



*Fig. 2.* Early stage of inflammation: the penis is coated by foamy mucus



*Fig. 3.* Diphtheroid pseudomembrane on the glans penis (medium severe stage)



*Fig. 4.* The ulcerated glans penis has become detached

consumption was also reduced. After temporary fluctuations, egg production became stabilized at a lower level. The egg infertility rate increased very rapidly, reaching 90% by 11 March, so that hatching had to be stopped.

On postmortem examination, the carcases uniformly showed pericarditis and peritonitis, with occasional deposition of yellowish fibrin in the changed areas. Petechial haemorrhages were found in pericardium, kidneys and pancreas.



*Fig. 5.* Subsided inflammation. About one third of the penis has become detached. Note chronic inflammation of the cloacal sphincter

One bird also had endocarditis. The spleen was generally swollen, with petechial haemorrhages on the capsule and occasionally necrotic foci appeared in the pulp. The testicles were as a rule normal-appearing, only a single gander had yellowish, pin-prick necrotic foci in the testes. Inflammation of the oviduct was often found in layers.

The mucous membrane of the cloaca was markedly red, with injected vessels, and was often covered by diphtheroid pseudomembranes and ulcerations, ranging in size from that of a pea to a bean. In several cases, the lumen was filled by a fibrinous–caseous substance of the cloaca, which extended into the abdominal cavity. A similar fibrinous, caseous-purulent substance filled the space under the prepuce of the ganders.

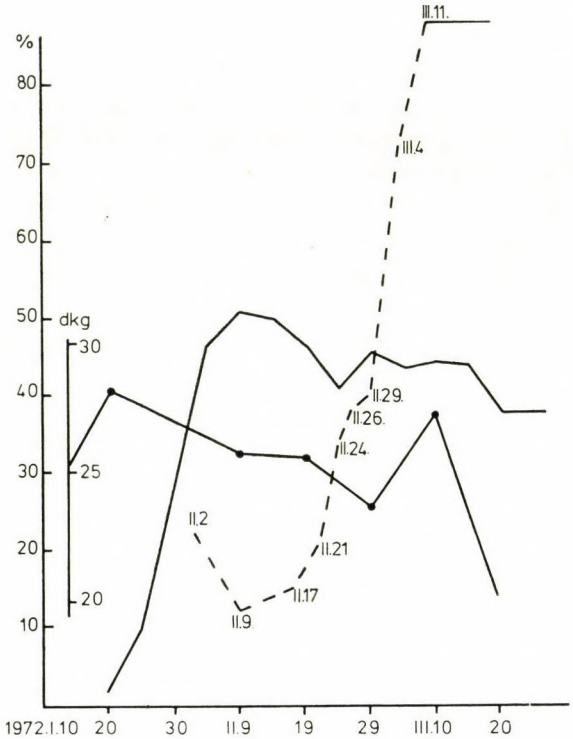


*Fig. 6.* Curve for course of outbreak in a large goose flock. Note the rapid rise in the egg infertility rate. ——— egg production; ·—·—· feed consumption; - - - - - - sterility

A Gram-negative mono-diplococcus was regularly isolated not only from the genitourinary tract, kidneys, testicles, mucous membranes of penis and cloaca, but also from the spleen, heart blood, and pancreas of the dead geese.

Attempts at classification of the isolate showed it to belong to the genus *Neisseria*. It is, however, distinct from *Neisseria gonorrhoeae*, being apparently a specific pathogen of geese. The properties of the new *Neisseria sp.* and its behaviour in infection experiments are the subject of another report.

## Therapy and Control

The following therapeutic and control measures were employed.

Each bird of the flock was examined individually and those layers which had a swollen cloacal region and thickened, palpable sphincter muscles as well as the ganders with ulceration or detachment of the male organ were culled, on the grounds that severe, chronic changes no longer respond to therapy.

The inflammatory lesions of the ganders were treated locally to prevent progression and suppuration. The penis was washed in disinfectant (2% Tego-51) solution and treated with an oxytetracycline powder. The sick ganders were isolated from the rest of the flock for 8—10 days. In certain cases, simultaneously with the local treatment, 5000 IU/kg penicillin was administered intramuscularly to all birds of the flock, including the apparently healthy ones. Flock treatment with antibiotic was deemed necessary because the disease was clearly contagious and bacteraemia was likely to occur as shown experimentally.

The isolates were regularly tested for antibiotic resistance, and the antibiotic producing the widest inhibition zone was used for therapy whenever possible.

At the beginning of the outbreak (when 5 to 10% of the ganders were involved), feed medication with ERRA-6 at 1% dietary level (600 g oxytetracycline per ton feed) for one- two-week periods, separated by a drug-free interval of one week, proved to be a successful therapeutic approach.

Convalescent birds do not develop immunity, whence reinfection of the ganders can occur on contact with the layers. Flock replacement by new ganders is therefore not advised because infection persists in the flock if recovery is incomplete and the new males also contract the disease. The libido of the spontaneously recovered or cured ganders was as a rule satisfactory; the minor decrease in egg infertility after a controlled outbreak remained within the limits of economic production.

The following measures are proposed for the prevention of spread and introduction of the disease:

Groups of infected, but recovered birds should be treated as a closed flock and culling of the affected individuals should be performed gradually.

The premises of the infected flock should be disinfected with 5% chlorine after a general cleaning.

Keeping of the birds in small groups, improvement of the hygienic conditions and prophylactic feed medication with oxytetracycline for one week during the laying seasons (every six week) are important preventive measures.

Periodical control examinations of the male organ of ganders at 2—3-week intervals are advised.

During the autumn stocking all layers and ganders should be examined individually for state of the reproductive organs and should be culled on the least suspicion of disease.

## Summary

A previously unknown, infectious venereal disease, causing great losses and greatly affecting reproduction, was observed in large goose flocks. The condition is characterized by a severe disease of the male organ of ganders and by an unusually high rate of egg infertility. The first symptom is inappetence, followed by inflammation of the cloaca, in male

birds by reddening and pseudomembranaceous inflammation of penis and cloaca. In severe cases prolapse of the penis is followed by its partial or complete detachment. In the infected flocks 10% of the diseased ganders died within a few weeks. A microorganism resembling *Neisseria gonorrhoeae*, but distinct from it, was isolated from lesions and organs of carcases and is therefore regarded as the causal agent. The symptoms and gross lesions of the goose venereal disease and proposed therapeutic and preventive measures are described in detail.

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Address of the authors: Dr. Iván SZÉP, Dr. Mária PATAKY, 2100 Gödöllő; Dr. Gyula NAGY, 1052 Budapest, Petőfi S. u. 3, Hungary.

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# AN INFECTIOUS INFLAMMATORY DISEASE OF CLOACA AND PENIS IN GEESE (GOOSE GONORRHOEA)

## II. PROPERTIES OF THE CAUSAL AGENT AND INFECTION EXPERIMENTS

By

Mária Pataky

Department of Physiology and Animal Health (Head: I. Szép), University of Agricultural Sciences, Gödöllő

(Received July 10, 1972)

The symptoms, epizootological course, gross lesions and experimental therapy of a goose venereal disease, caused by a *Neisseria*-like microorganism, were reported previously (Szép et al., 1971). Studies on the morphological and biochemical properties and antibiotic sensitivity of the causal agent and the results of infection experiments are described in this paper.

### Materials and Methods

*Bacterium strains* isolated from various large goose flocks were designated with the initials of their place of origin as O, Hsz, Hn, Z, R, F and T.

*Growth media.* Common broth, common agar plates containing 1% glucose and agar plates containing 5% sheep blood were used for isolation. The carbohydrate decomposing activity of the isolates was examined in liquid media, containing 1% glucose or glucoselike alcohols — which are seen in the Table I — and an appropriate pH indicator. Gas production was examined in Durham tubes placed on the bottom of the culture tubes.

*The motility* of the isolated organisms was examined in 0.2% agar columns.

*Sensitivity to antibiotics* tested by means of the Resitest-disc technique. 0.1 ml of a 24-hour broth culture was evenly spread on the surface of a glucose-agar plate and after appropriate drying in the incubator, the antibiotic-containing discs were placed on the agar surface at equidistant spaces. The diameters of the inhibition zones were determined after an incubation for 24 hours at 37°C.

*Samples.* Exudation from the affected penis and cloacal mucosa and certain organs of diseased or dead ganders were used as infectious material.

## Morphological, biochemical and staining properties of the isolates

One and the same microorganism was isolated from the penis and cloacal mucosa of all diseased layers and ganders, from the testicles of the ganders and from the spleen, heart blood, pancreas and kidney of carcases.

First isolations were equally successful in glucose agar and blood agar within 24 hours at 37°C. The colonies were very small, extremely delicate and transparent, sometimes showing opalescence in transmitted light; they were generally round, some showing a slight granulation and delicate lobulation at the margins, especially after 2—3 days. The microorganisms were Gram negative; on staining with Löffler's methylene blue they could be identified under the microscope as small mono- or diplococci. The adjacent surfaces of each pair were straight, thus the shape of the cocci reminded of coffee beans. The organisms were about 0.5 $\mu$ in diameter. The same cocci were identified in smears taken from the root of the penis of diseased birds and stained with Löffler's technique.

Bacterial growth was poor in common broth. Successful transfers were made after storage for 2—3 weeks in the refrigerator or at room temperature. The cultures showed neither haemolytic activity nor motility. They decomposed glucose, sucrose, maltose, salicin and — after 48 hours — also trehalose; the fermentation of glucose and salicin was accompanied by gas formation. Lactose, sorbitol, mannitol, raffinose, inulin, dulcitol, adonite, arabinose and glycerol were not decomposed by the strains and lithmus milk showed no change (Table I).

## Antibiotic sensitivity

Morphologically and biochemically identical isolates from carcases of spontaneously died or freshly killed birds originating from various flocks were tested for resistance to penicillin, streptomycin, aureomycin, terramycin, chloramphenicol, tetracyn, neomycin, erythromycin and polymyxin-B. All strains proved to be resistant to Superseptyl, the single sulphonamide tested. Inhibition zones 20—30 mm in diameter were found with all antibiotics tested (Table II). The degree of sensitivity to certain antibiotics varied slightly with the flock, but it was uniformly high to penicillin.

## Infection experiments

The following infection experiments were carried out to substantiate the pathogenic nature of the isolates and the infectious nature of the disease:

*a)* Broth culture was rubbed into the mucous membrane of the penis of healthy ganders.

**Table I**

Carbohydrate decomposition and other properties of the isolates

| Carbohydrate | 24 hours | 48 Hours |
|---|---|---|
| Glucose | acid + gas formation | acid + gas formation |
| Lactose | negative | negative |
| Sucrose | acid formation | acid formation |
| Maltose | acid formation | acid formation |
| Trehalose | negative | acid formation |
| Sorbitol | negative | negative |
| Mannitol | negative | negative |
| Raffinose | negative | negative |
| Inuline | negative | negative |
| Adonite | negative | negative |
| Dulcitol | negative | negative |
| Arabinose | negative | negative |
| Salicin | acid + gas formation | acid + gas formation |
| Glycerol | negative | negative |
| Haemolytic activity | negative | negative |
| Motility | negative | negative |
| Lithmus milk | negative | negative |

**Table II**

Antibiotic sensitivity of the isolates

| Antibiotic | Diameter of inhibition zone in mm for strains | | | | | | |
|---|---|---|---|---|---|---|---|
| | O | Hsz | Hn | Z | R | F | T |
| Penicillin | 30 | 28 | 28 | 22 | 22 | 26 | 28 |
| Streptomycin | 23 | 22 | 23 | 20 | 20 | 22 | 23 |
| Aureomycin | 28 | 23 | 22 | 20 | 20 | 23 | 21 |
| Terramycin | 25 | 25 | 30 | 20 | 22 | 23 | 23 |
| Chlorocid (Chlortetracycline) | 28 | 26 | 30 | 20 | 23 | 25 | 25 |
| Tetracyn | 25 | Ø | Ø | 17 | 20 | 22 | 23 |
| Neomycin | 26 | 30 | 30 | 30 | 25 | 26 | 26 |
| Erythromycin | 28 | 25 | 25 | 20 | 23 | 24 | 25 |
| Polymyxin-B | 20 | 20 | 20 | 18 | 17 | 18 | 19 |
| Superseptyl | Ø | Ø | Ø | Ø | Ø | Ø | Ø |

*b)* Organ suspensions prepared from homogenized testicle and kidney of ganders slaughtered in advanced stage of disease were applied intraperitoneally and by subcutaneous infiltration to the cloacal region.

*c)* Blood obtained from birds killed in advanced stage of disease was applied intraperitoneally and by subcutaneous infiltration of the cloacal region to healthy ganders and layers.

*d—e)* A healthy layer was placed with an experimentally infected gander and *vice versa*. The male and female birds of groups *e* and *d* were separated from one another after one week. All experimental birds (groups *a—e*) were kept in individual cages.

*Latency period and symptoms.* The cloacal region showed no change after 3 days, but after 5 days it exhibited swelling, rise of local skin temperature, and reddening of the mucous membrane. The penis of the ganders simultaneously exhibited reddening at the root and was coated by a viscous mucus; in some cases, the male organ was difficult to expose. The healthy ganders and layers exposed to infection by contact also contracted the disease and began to show the above symptoms after one week; the cloaca and penis were affected to different degrees, but never as severely as in the naturally diseased geese.

## Blood tests

The blood of experimentally infected birds was tested by tube agglutination on three occasions, 6, 8 and 16 weeks after the infection. The results were positive throughout at serum titres ranging from 160 to 320; values for 6 and 16 weeks were practically identical.

## Discussion

Practical observations during outbreaks and laboratory studies of the isolated coccus strains strongly suggest that a bacterium species belonging to the genus *Neisseria* should be regarded as the primary causal agent of the infectious inflammatory disease of penis and cloaca in the large goose flocks under study.

The light-microscopic appearance and colony morphology of the cocci justify their classification as a yet unknown species of *Neisseria*. Although the epizootological course and mode of infection of the goose venereal disease correspond with human gonorrhoea, the biochemical behaviour of the strains is distinct from that of *Neisseria gonorrhoeae* and of all other *Neisseria* species known from systematic descriptions *(N. sicca, N. intracellularis, N. flava, N subflava, N. perflava)*.

The clarification of the true importance of the new *Neisseria* species, which seems to be a specific pathogen of geese, requires further investigation.

## Summary

A bacterium species belonging to the genus *Neisseria* was isolated as the primary causal agent of a goose venereal disease not observed previously. The disease affects the cloaca and in ganders the penis. The epizootological course, the mode of infection and the gross lesions corresponded with those of human gonorrhoea, but in biochemical tests the isolates behaved differently from *Neisseria gonorrhoeae* and all other known *Neisseria* species *(N. sicca, N. intracellularis, N. flava, N. subflava, N. perflava)*.

The clarification of the importance of the new *Neisseria* species, which appears to be a specific pathogen for geese, warrants further study.

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The address of the author: Dr. Mária Pataky, 2100 Gödöllő, Hungary

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# ERECTION APPARATUS OF THE COPULATORY ORGAN OF GANDERS AND DRAKES

By

E. GUZSAL

Dept. of Anatomy and Histology (Head: Prof. GY. FEHÉR)
University of Veterinary Science, Budapest

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Erection of the copulatory organ of male animals takes place in consequence of the engorgement of the cavernous spaces of the penis with blood in mammals and with lymph in birds. Although the avian copulatory organ is either rudimentary, as in cocks, or well developed, as in the gander and drake, the mechanism of erection and the structure and function of the avian erectile apparatus were found to be essentially similar in the species studied so far.

The role of lymph in the erection of the avian copulatory organ has long been known (ECKHARD, 1870; MÜLLER, 1908; LIEBE, 1914), but certain reproduction biological and pathological problems arising under the conditions of large-scale poultry farming have recently revived interest in details of morphology and function. Many authors, recognizing the importance of the problem (NISHIYAMA, 1955; LAKE, 1957; NISHIYAMA and OGAWA, 1961; KOMÁREK, 1969; MARVAN, 1969; FEDER, 1970, KNIGHT, 1967, 1970), investigated the structure and function of the copulatory organ and corpus vasculare (LIEBE's "gefässreicher Körper", further on referred to by NISHIYAMA's term as vascular body). "The lymph or a fluid similar to lymph" (NISHIYAMA) is generated in the vascular body and from there it flows through a wide duct into the cavernous spaces of the copulatory organ.

Despite extensive studies on the topic, no literary data are available on the erection apparatus of aquatic birds, nor on the electron microscopic structure of the vascular body; also, several details of lymph filtration and of the mechanism of erection and relaxation are still poorly understood.

## Materials and Methods

The reproductive organs of the gander and drake were studied during the active period of sexual function. Fresh and formalin-fixed organs were processed. Specimens for light and electron microscopic study were secured immediately after killing the birds by bleeding. Ten percent neutral formalin, Helly's fixing solution or glutaraldehyde and osmium tetroxide were used for fixation. Sections cut from paraffin-embedded specimens were stained with haematoxylin and eosin, van Gieson's stain or resorcine and fuchsin. Sections for electron microscopy were prepared from Durcupan-embedded (FLUKA)

specimens with a Reichert Om U2 ultramicrotome and were counterstained with uranyl acetate and lead citrate. The electron micrographs were prepared with a TESLA BS type 613 apparatus, in the Electron Microscopy Laboratory of the University of Veterinary Science.

Roentgenograms of the vascular net were prepared after the injection of Novobarium and corrosion preparations after the injection of Technovit (KULZER).

## Results

In the quiescent condition, the penis of both gander and drake lies folded in a thin connective tissue "capsule" on the ventrolateral wall of the cloaca. The outer surface of the capsule is smooth, while inside it a loose connective tissue, admixing with elastic fibres, is found, which is connected with the penis. Thus the quiescent penis is not visible through the cloaca; its opening *(ostium penis)* is, along with the *fossa ejaculatoria*, surrounded by mucosal folds. The supporting structure of the folds is an elastic cartilage, a bent, troughlike lamella, whose base associates with the ventral wall of the cloaca in the region of the urodaeum, while its two edges extend into the folds. At the

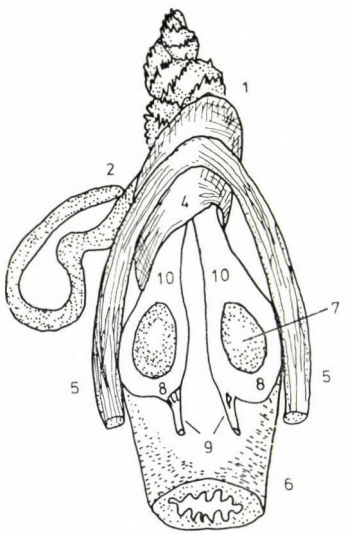


*Fig. 1.* Scheme of the erection apparatus of the penis in aquatic birds (ventral view, with the penis slightly erected). 1, basis penis; 2, apex penis; 3, flexura penis; 4, cartilaginous lamella; 5, m. retractor penis; 6, rectum; 7, vascular body; 8, lymph sinus; 9, lymph vessels; 10, lymph duct

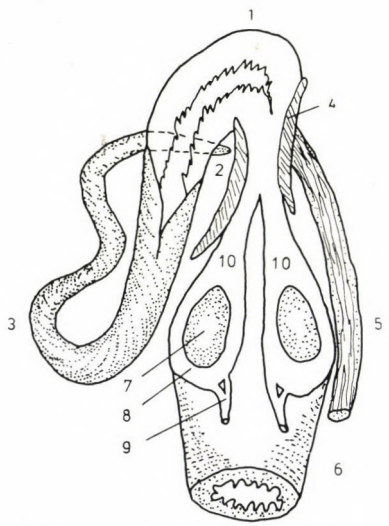


*Fig. 2.* Scheme of the erection apparatus of the penis in aquatic birds (ventral view, with the region of the penis base exposed). Symbols as in Fig. 1

caudal end of the cartilaginous lamella begins the base of the penis, nearby which on the left is attached the apex of the penis, and along its outer surface extends the cylindrical retractor muscle of the penis (m. retractor penis, s.-cloacae), about 4 mm in diameter.

The penis consists of two, almost equally long, adjacent portions, linked with one another by a loose connective tissue (*suspensorium penis* according to KOMÁREK). The tube-shaped first portion *(portio proximalis)* extends from

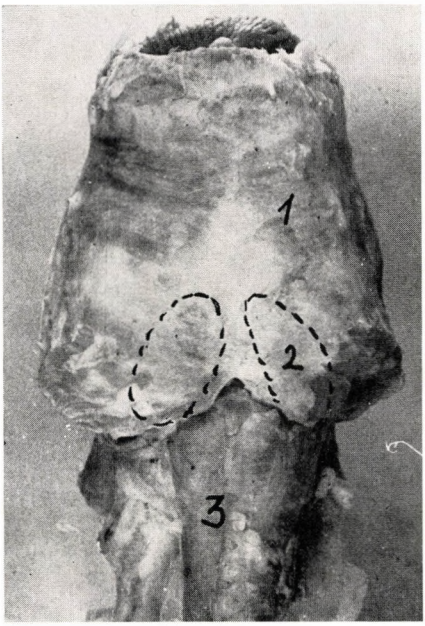


*Fig. 3.* Cloacal region in ventral view (gander). 1, cloacal sphincter muscle; 2, (framed) localization of vascular bodies; 3, rectum

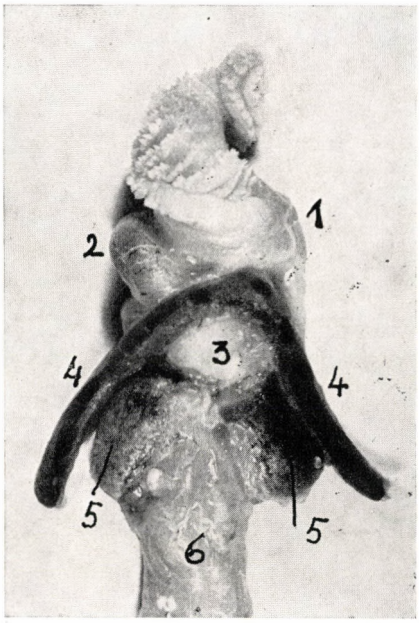


*Fig. 4.* Cloacal region in ventral view (gander), after removal of the cloacal sphincter muscle and with penis slightly erected. 1, basis penis; 2, apex penis; 3, cartilaginous lamella; 4, m. retractor penis; 5, right and left vascular bodies; 6, rectum

the *basis penis* to the *flexura penis*, its mucosal coat is continuous with the mucous membrane of the cloaca and it is covered by a keratinized stratified squamous epithelium (*pars cutanea*, KOMÁREK); as the spaces of the cavernous tissue are present in the wall of the first portion, it may also be termed as *pars cavernosa*. The second portion of the penis, *portio distalis* (*pars extrema penis* according to KOMÁREK), extends from the flexura to the *apex penis* and contains glands *(pars glandularis)*, but no cavernous spaces are demonstrated. The apex of the penis attaches to the cartilaginous lamella. At erection, the proximal portion of the penis protrudes from the cloaca by winding spiral movements, whereby the mucous membrane and the helical *sulcus ejaculatorius*

are everted to the outer surface, and the more compact distal portion fills the inside of the proximal portion; since the apex of the penis is attached to the cartilage, the erectile penis can only protrude from the cloaca at half length.

The vascular bodies, *corpus vasculare dextrum et sinistrum*, localize on the ventrolateral surface of the cloaca, roughly at the level of the coprodaeum, and their longitudinal axes would enclose an acute angle if they were prolonged

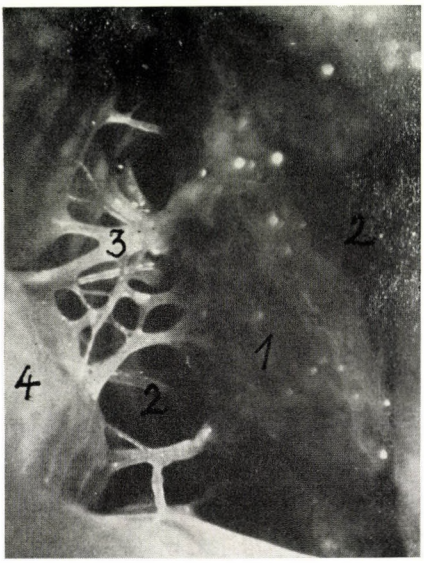


*Fig. 5.* Vascular body of gander after cutting open the lymph sinus. 1, vascular body; 2, lymph sinus; 3, trabecules; 4, cloacal sphincter muscle

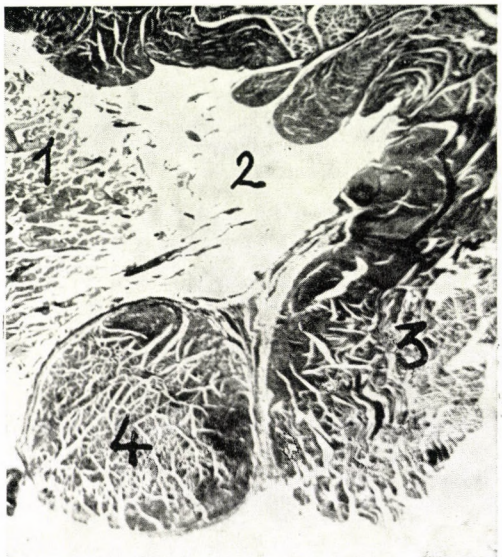


*Fig. 6.* Cross-section of the vascular body region (H. and E., hand magnifying lens). 1, vascular body; 2, lymph sinus; 3, cloacal sphincter muscle; 4, m. retractor penis

in the caudal direction. The vascular bodies are plum-shaped, red-coloured, of compact structures, $20 \times 10$ mm in size, which cannot be seen or approached unless the *m. sphincter cloacae* is cut open.

Dorsally, the vascular body is closely associated with the cloacal adventitia, the ductus deferens and ureter passing between them towards the urodaeum; the rest of the surface of the vascular body is surrounded by a peripheral lymph sinus (*cavum lymphaticum*, "Lymphbildungsraum", LIEBE). The lymph sinus or cavity is surrounded by muscles, the *m. sphincter cloacae* and *m. retractor penis*, and its wall is formed by thin connective tissue and endothelium, which are associated with the fascia of the muscles. Many thin, elastic strands *(trabeculae)* transverse the space between the vascular body and the wall of the lymph sinus. The vascular bodies are linked with the cavernous spaces of the penis by a wide duct, the connecting portion *(ductus*

*lymphaticus);* the bilateral connecting portions communicate with each other under the cartilaginous lamella. The system of peripheral lymph sinuses and the cavernous tissue spaces of the penis are lined by endothelium. The intracavernary tissue nearby the base of the penis, contains corpuscles of Herbst.

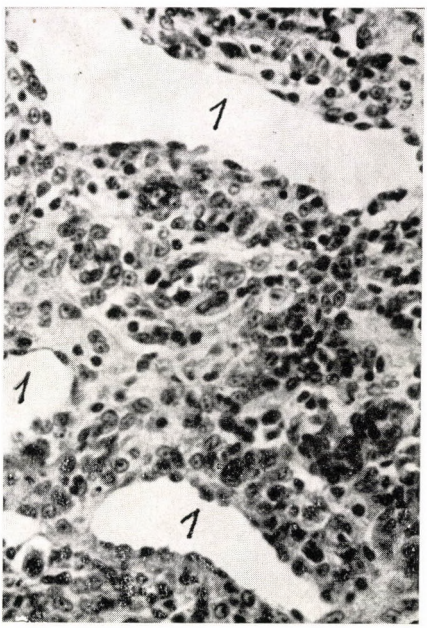


*Fig.* 7. Light micrograph of vascular body (H. and E.). 1, lymph spaces




*Fig. 8.* Marginal detail of vascular body (resorcine and fuchsin stain). 1, lymph spaces; 2, lymph sinus; 3, trabecula

The vascular body is supplied by the thick, tortuous internal pudendal artery *(a. pudenda interna)* which divides into 5 or 6 branches in the dorsal (cloacal) part of the structure. Each branch gives off radial tufts of undulating small arteries and arterioles; the space between the vessels is filled by a dense mesh of capillaries, which join to form veins in the dorsal part of the vascular body. The internal pudendal vein *(v. pudenda interna)* leaves the vascular body side by side with the artery. The observation that in corrosion preparations prepared with a coloured substance the internal pudendal vein was much paler than its arterial counterpart lead to the conclusion that the coarser granules of the colouring matter did not pass on to the vein, being trapped in the narrow connecting branches, the metarterioles; this means that no arteriovenous anastomoses exist between the larger vessels even in the quiescent condition.

The connective tissue supporting the structure of the vascular body

contains fibroblasts, fibrocytes and reticulum cells. The greater part of the fibres are collagen and reticulum fibres (tropocollagen); the latter form a dense network nearby the fibroblasts and basement membranes. The vascular body also contains many elastic fibres, which extend into the trabeculae traversing the lymph sinus. The arteries (small arteries and arterioles) have a thick wall and a narrow lumen; both the internal and external elastic lamina are well developed, especially the latter, which is more pronounced than usually. The veins are thin-walled, with a wide lumen, and the adventitia contains many elastic fibres. Under the light microscope, the lumina of the capillary vessels were so narrow that they could scarcely be recognized. The endothelium-lined lymph spaces, which communicate with one another and the lymph sinus, are characteristic and conspicuous parts of the vascular body. The trabeculae are of similar tissue structure to the vascular body and are coated by endothelium. Many lymph vessels originate from the lymph sinus.

Electron micrographs have shown that the capillaries of the vascular body are so-called muscle-type vessels, lined by uninterrupted endothelium. The lining endothelial cells overlapped in the narrow, constricted capillary. Apart from pinocytotic vesicles, corresponding with pinocytotic invaginations on both surfaces, there were many cytoplasmic organelles: a granular endoplasmic reticulum, a small Golgi apparatus, a medium amount of mitochondria with a few cristae, lysosomes, many free ribosomes (polysomes) and filaments were seen inside the cells. The luminal side of the endothelium was relatively smooth, only a narrow process or "marginal fold" being extended at the end of the cells. At the intercellular junctions there were desmosomes; a "zonula occludens" was rarely seen. The nuclei were either oval or slightly flat, depending on the shape of the cell. The endothelium was surrounded by a basement membrane and by adventitial cells (pericytes).

In accordance with light micrographs, the predominant connective tissue cells proved to be fibroblasts and only very few fibrocytes and reticulum cells were present among them. The intercellular space was filled by a relatively loose mesh of collagen and elastic fibres and tropocollagen filaments.

Many unmyelinated nerve fibres and autonomous plexuses were also seen in the interstitial tissue of the vascular body. Plexuses were found not only nearby vessels, but also farther removed.

Several synapses were seen at the marginal parts of Schwann cells. The synaptic axon parts contained, apart from a few mitochondria, many vesicles which were of two types, clear ones, about 250—300 Å in diameter, and dense-cored vesicles, about 1000 Å in diameter; both types occurred in each synapsis, but at varying proportions.

The endothelial cells lining the lymph sinus system were similar in structure to those lining the capillaries and the endothelium was separated from the connective tissue by a basement membrane.

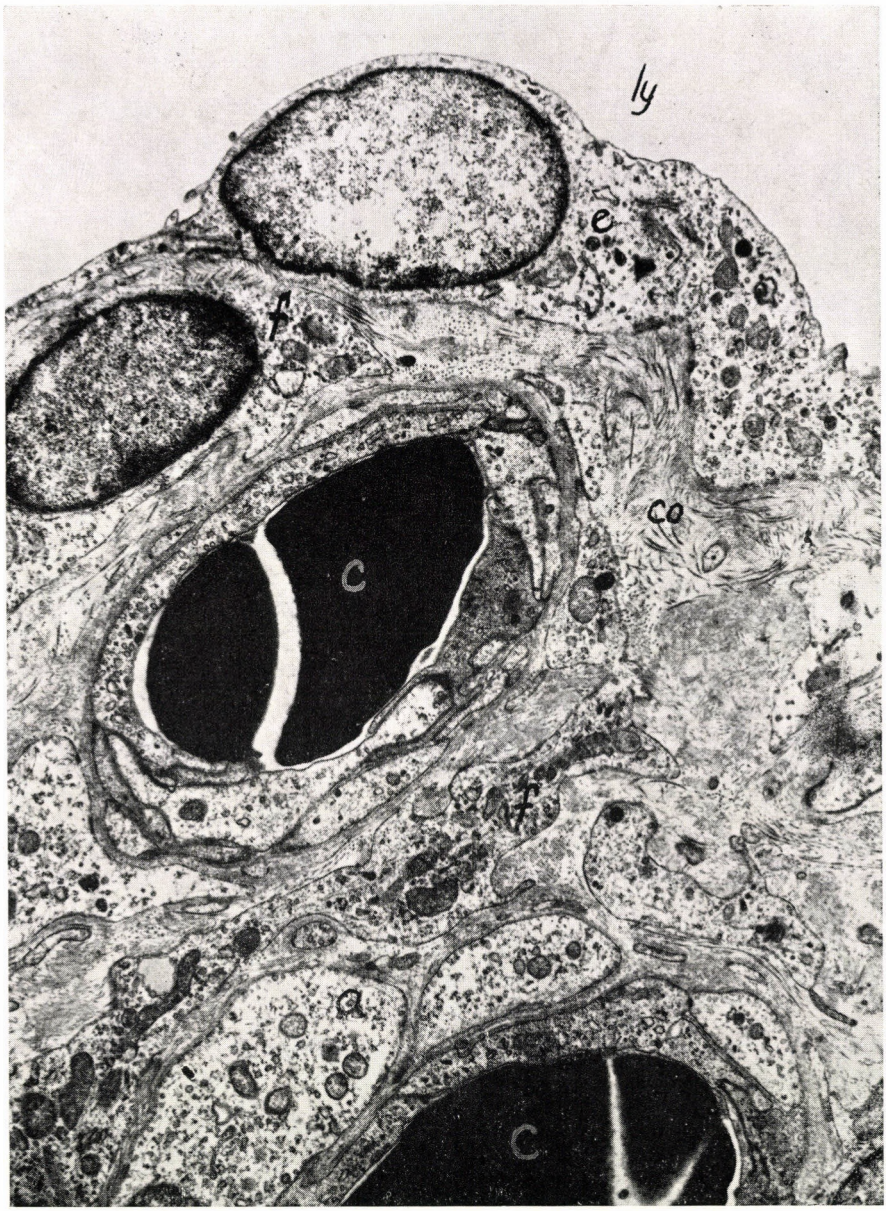


*Fig. 9.* Vascular body from gander. An endothelial cell (e) separates vascular body surface from lymph sinus (ly). Note cross-sections of capillaries (c), adventitial cells (a), details of fibroblasts (f) and collagen fibres (co) inside vascular body. The lumina of capillaries contain erythrocytes. Electron micrograph ×10,186

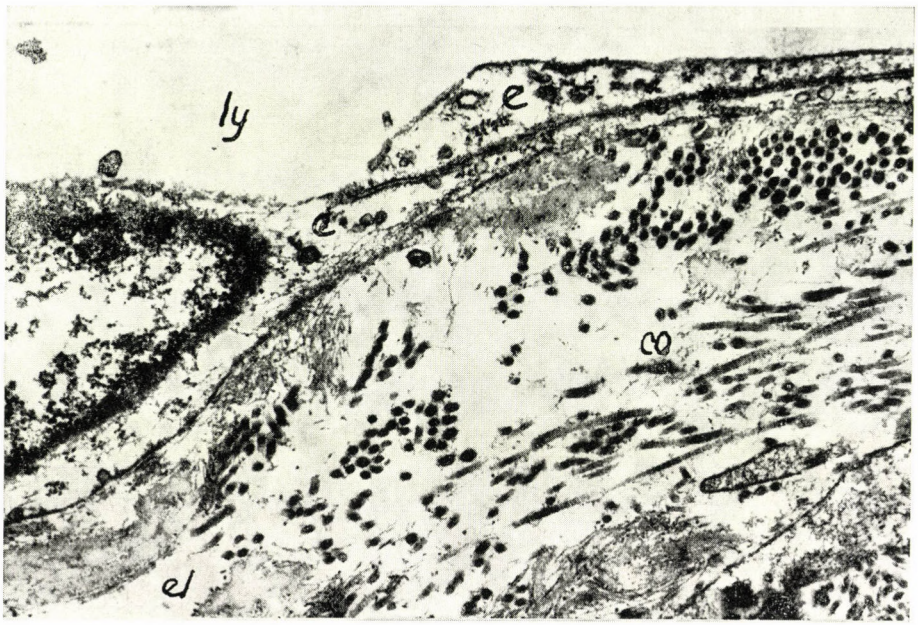


*Fig. 10.* Vascular body of gander. The lymph sinus (ly) is lined by endothelial cells, beneath which there are collagen (co) and elastic (el) fibres. Electron micrograph ×24,750

## Discussion

A nomenclatural confusion is still existing in respect of the avian copulatory organ. The male organ of gallinaceous birds does not protrude from the cloaca at erection *(phallus non protrudens)*, whereas that of aquatic birds does, being accordingly termed as *phallus protrudens*, *fibrous penis* or simply *penis*.

The various organs or organ parts participating in the complex mechanism of erection are termed collectively as the erection apparatus. The components of the latter are the paired vascular bodies, the system of lymph sinuses, the vessels, the muscles of the cloaca and the cavernous spaces of the penis. The fluid regarded as lymph, playing the primary role in erection, is generated in the vascular body.

Under the influence of a sexual stimulus, which probably generates a parasympathetic impulse, muscle relaxation takes place in the arteries and precapillary arterioles, resulting in distension of the lumina. Thus a large amount of blood is pushed into the capillaries at a high pressure, bringing about dilatation and thinning of the capillary wall, through which blood plasma becomes extravasated into the interstitial space, owing to an increased hydrostatic pressure. Simultaneously the venous drainage tends to decrease, prob-

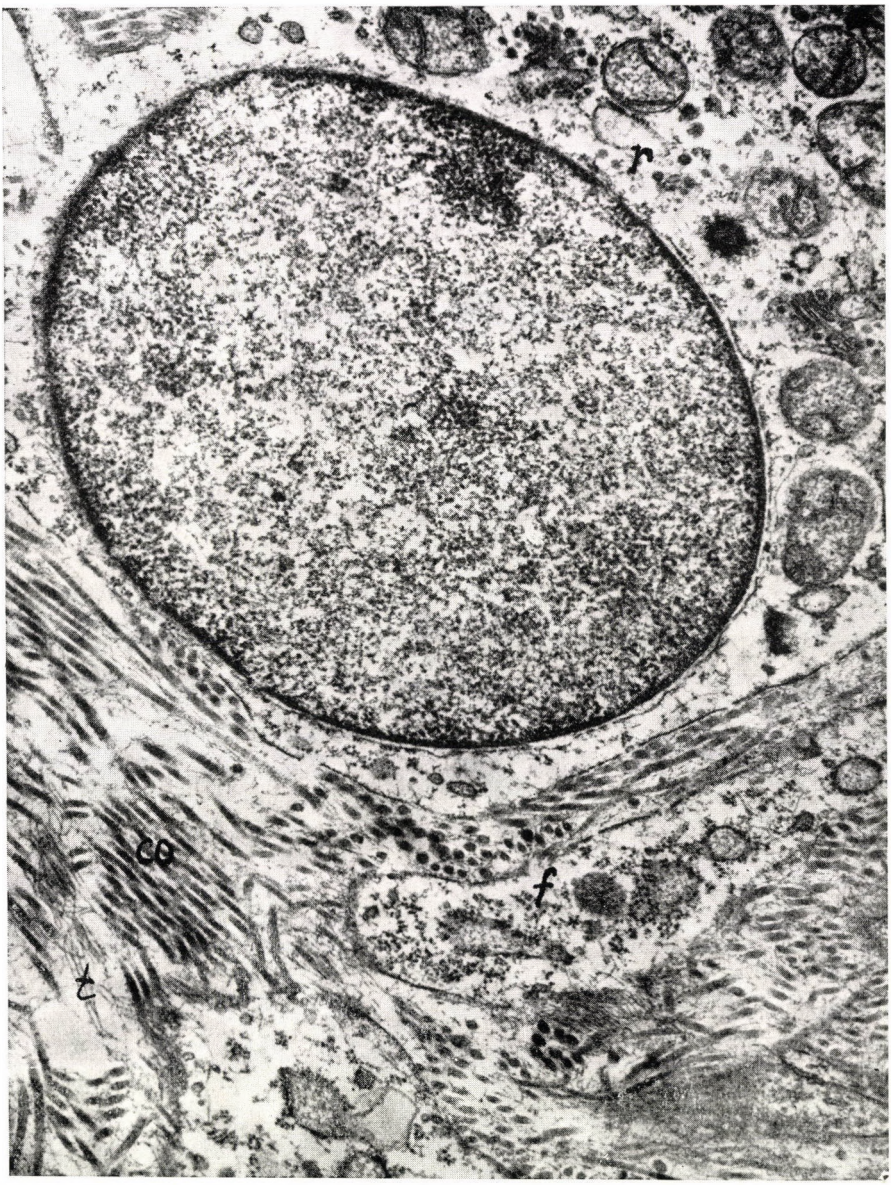


*Fig. 11.* Vascular body of gander. Reticulum cell (r), detail of a fibroblast (f), tropocollagen filaments (t) and collagen (co) fibres. Electron micrograph ×21,800

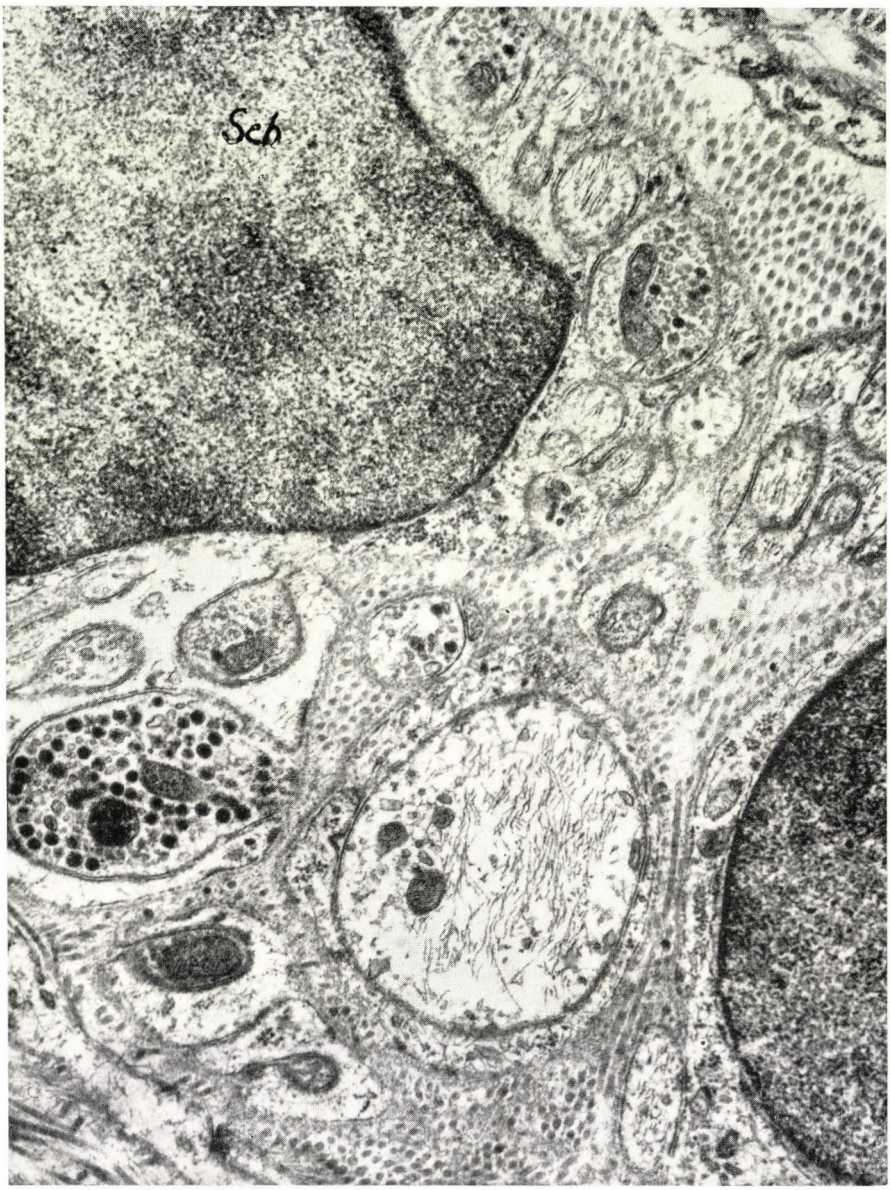


*Fig. 12.* Vascular body of gander. Autonomic plexus with synpases. Sch, Schwann cell. Electron micrograph ×21,800

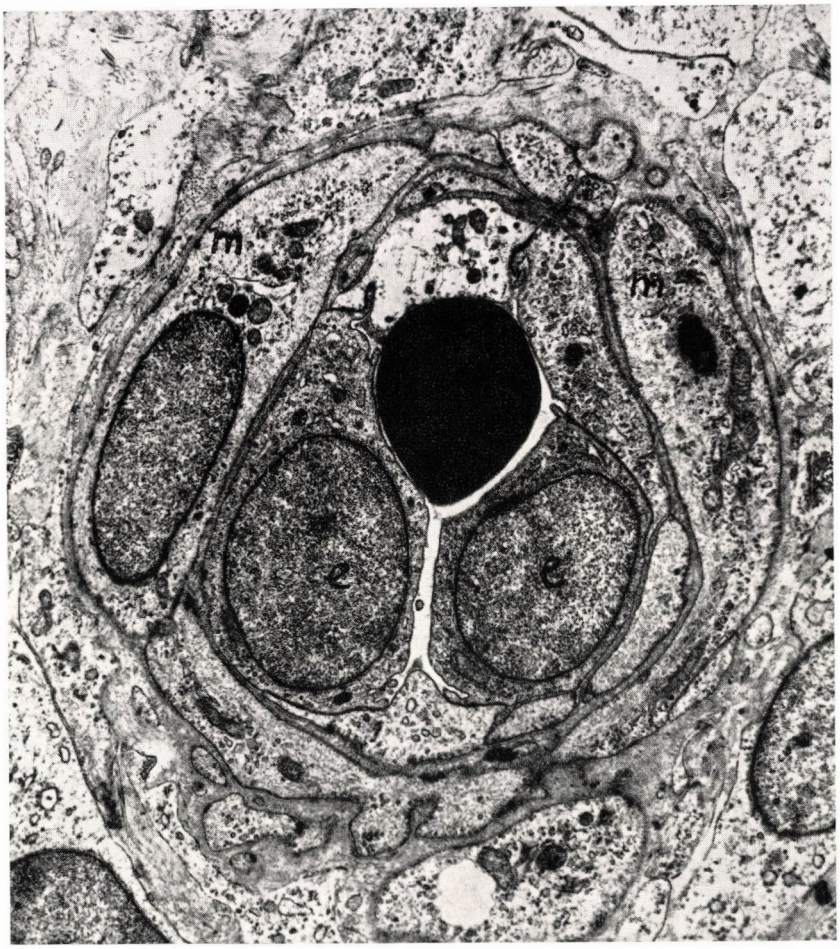


*Fig. 13.* Vascular body of gander. Precapillary arteriole. (e) endothelial cells, (m) smooth muscle cells. Electron micrograph ×9260

ably as a result of compression of the thin-walled veins. Owing to engorgement of the interstitial tissue, the plasma filtrate or lymph is further filtered into the lymph spaces and lymph sinuses, from where the filtration pressure and the surrounding muscles press it through the lymph channels until it fills the cavernous spaces of the penis. The channels connecting the lymph sinuses with the penis localize inside the troughlike cartilage lamella described in this paper and as the penis arise at the caudal end of the cartilage, the lymph reaches them without a notable decrease in pressure.

The relaxation following upon the ejaculation of semen being a passive process, takes place slower than erection, which is an active process. When the sexual stimulus ceases, either the parasympathetic impulse disappears or the

sympathetic influence becomes predominant so that the muscles of the arteries and precapillary arterioles gradually contract, and the amount and pressure of blood pushed into the capillaries reduce until the extravasation of lymph across the capillary wall ceases. As the cloacal sphincter muscle which surrounds the lymph sinus also becomes relaxed or at least its tone decreases, the sinus distends and the lymph flows back from the penis and is drained off through

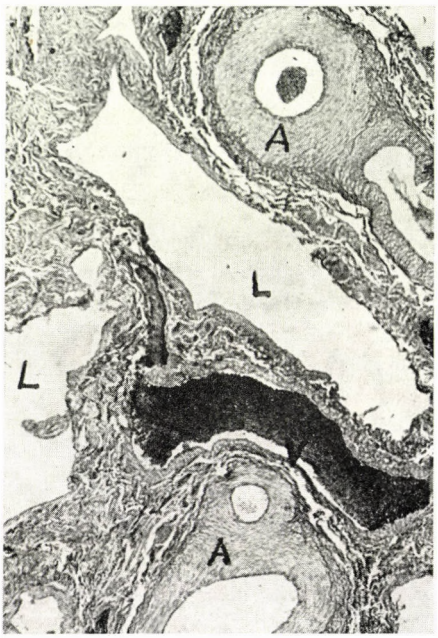


*Fig. 14.* Vascular body of gander. Detail of the cranial end. H. and E. A, artery; V, vein: L, lymph vessel

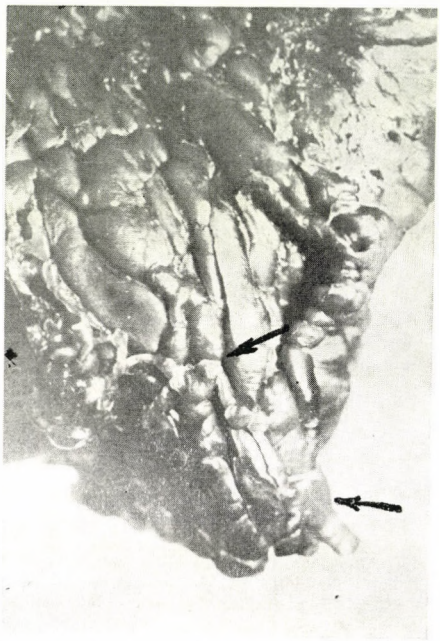

*Fig. 15.* Cranial end of vascular body from gander. Corrosion preparation. (The arrows point to lymph vessels)

the lymph vessels. The latter contain densely arranged valves which arise from the medial and cranial parts of the lymph sinus and have well-developed muscles at their origin to act as a sphincter during the period of erection.

According to KOMÁREK, after erection a paired muscle *(m. retractor penis)* attaching at the apex of the penis retracts the male organ to its quiescent position. According to present studies, the attachment of the said muscle is at the ventral surface of the cartilage lamella and thus having no direct association with the penis, it cannot possibly act as the latter's retractor. It actually plays this role in the rudimentary copulatory organ of cocks, because in male gallinaceous birds the ventral part of the cloaca becomes everted along with the phallus at erection and after ejaculation the muscle retracts the structures of this region to the quiescent position. In aquatic birds the function

of the muscle is similar but, owing to the different structure of the copulatory organ it acts only on the base of the penis, *viz.*, on the ventral part of the cloaca. The penis itself is retracted by spiral connective tissue fibres, above all elastic fibres, which are taut during erection and by the fibres of the suspensorium penis after decrease or cessation of the erectile forces. Simultaneously the lymph gradually returns from the penis to the lymph sinus, from where, as mentioned in the foregoing, the excess lymph is drained through the lymph vessels.

## Summary

The structures collaborating in the erection apparatus of male birds are the vascular bodies, the system of lymph sinuses, the cloacal muscles and the cavernous tissue of the penis. The paired vascular body localizes at the ventral surface of the cloaca and the wide lymph sinus surrounding it communicates with the penis through connecting lymph channels. The vascular body is supplied by the internal pudendal artery, which divides to form a dense mesh of capillaries inside it. The capillaries and lymph sinuses are lined by uninterrupted endothelium. On a sexual stimulus, the muscles of the arteries and precapillary arterioles relax, the lumina distend and the blood pushed abruptly into the capillaries causes them to distend and to extravasate lymph as a result of the increased hydrostatic pressure. The lymph extravasated into the lymph sinus is pressed further into the penis by filtration pressure and by the cloacal sphincter muscle, which surround the lymph sinus. The reverse process is taking place during relaxation. The penis is retracted to its quiescent position by elastic fibres which are taut during erection, the so-called *m. retractor penis* being responsible only for the retraction of the ventral cloacal portion and the penis base. The lymph returns from the penis to the lymph sinuses, from where it is drained off by the lymph vessels.

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Address of the author: Dr. Ernő Guzsal, 1078 Budapest, Landler J. u. 2, Hungary

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# DEVELOPMENT OF THE INTESTINAL FLORA IN SUCKLING PIGS

By

GY. SINKOVICS and B. JUHÁSZ

Department of Animal Hygiene (Head: Prof. F. KOVÁCS),
University of Veterinary Science, Budapest,
and Institute for Animal Breeding (Director: J. KESERÜ), Herceghalom

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The changes induced by certain factors in the intestinal flora of healthy pigs have extensively been studied (SMITH, 1959; PESTI, 1964; HILL and KENWORTHY, 1970; MORISHITA and OGATA, 1970) and important observations have been made above all on bacterial digestive-tract diseases (KENWORTHY and CRABB, 1963; SMITH and JONES, 1963; SOJKA, 1965; NIELSEN et al., 1968; BARNUM, 1971; BERGELAND, 1972).

However, information has been scanty on the development of the intestinal bacterial flora, although the knowledge of the bacterial processes taking place in the gut of normal pigs is an essential prerequisite of the understanding of neonatal digestive organ diseases. To obtain more information on the problem, the development of the gut flora was examined in healthy pigs, with special regard to related studies by others (WILBUR, 1959; PESTI, 1962).

## Materials and Methods

The litters of two Large White sows were used in the experiments. Two piglets were killed by bleeding at each time between 2 hours and 12 days after birth, as indicated in Table I. Accordingly, the digestive tracts of two animals from one and the same litter were examined at one time. The techniques of abdominal incision, sampling and processing were the same as described previously (KOVÁCS et al., 1972), except that samples were only taken from stomach, jejunum, ileum and colon, for according to literary data and our own experience, in healthy animals the composition and proportions of the bacterial population are similar in the different segments of the large intestine.

Some samples obtained from very young animals were less than 1 g in weight; in such cases a correction factor was used for evaluation.

Coliforms were isolated on the selective McConkey agar (Difco B 75); whereas lactobacilli under anaerobic conditions, using an atmosphere containing 20% $CO_2$ and 80% $N_2$; the vegetative and spore-bearing forms of clostridia were isolated as described in a previous publication ((SINKOVICS, 1972).

**Table I**

Bacterial flora in the alimentary tract

| Age of piglets | | | Stomach | | | | | | Duodenum | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | L | S | C | Cl | Sp | pH | L | S | C | Cl | Sp |
| | in hours | 2 | 3.0 | 5.6 | 1.0 | 2.0 | — | 5.7 | 3.0 | 5.2 | 1.6 | — | — |
| | | 6 | 4.8 | 7.5 | 5.9 | 6.6 | 2.0 | 5.0 | 4.3 | 6.2 | 6.0 | 5.2 | 2.7 |
| | | 18 | 8.1 | 4.9 | 4.6 | 3.6 | 3.5 | 3.8 | 7.3 | 5.1 | 5.7 | 3.7 | 1.3 |
| | in days | 1.5 | 7.7 | 6.7 | 2.0 | 7.0 | 1.3 | 3.9 | 6.6 | 4.5 | 3.8 | 6.4 | 0 |
| | | 2 | 7.6 | 5.5 | 2.4 | 2.6 | 1.0 | 3.9 | 6.8 | 5.0 | 4.4 | 4.1 | 1.3 |
| | | 4 | 8.2 | 5.7 | 4.7 | 3.4 | 0.5 | 3.9 | 6.5 | 4.8 | 4.6 | 3.1 | 1.2 |
| | | 5 | 8.4 | 5.9 | — | 4.4 | 1.0 | 3.6 | 6.9 | 5.5 | 3.5 | 3.9 | 1.0 |
| | | 8 | 8.5 | 6.0 | 4.7 | 5.2 | 3.2 | 4.5 | 6.9 | 4.9 | 5.0 | 4.8 | 1.5 |
| | | 9 | 9.0 | 4.9 | 1.2 | 5.4 | 3.5 | 3.8 | 7.9 | 4.4 | 1.0 | 1.5 | 1.0 |
| | | 11 | 8.7 | 5.2 | 3.0 | 4.7 | 2.7 | 4.0 | 7.9 | 6.3 | 4.2 | 4.6 | 0.8 |
| | | 12 | 8.5 | 5.0 | 2.2 | 5.7 | 2.8 | 3.4 | 6.5 | 3.2 | — | 4.5 | 1.9 |

No attempt was made in the present study at isolation of non-spore-forming anaerobic organisms, because KENWORTHY and CRABB (1973) and FULLER and LEV (1964) showed them to occur in very low counts, if at all, in the gut of healthy suckling pigs.

## Results

The results of bacteriological examinations on stomach and intestinal segments of piglets killed at different points of time after birth are shown in Table I. Each numerical value represents the mean of the logarithms of germ counts determined in two pigs killed at one time.

Attempt was also made to isolate bacteria from the gut of piglets killed immediately after birth. Samples taken with sterile swabs transferred to media containing no bacteriostatics, *viz.*, RCM plate and broth. The media were incubated under aerobic and anaerobic conditions, but no bacterial growth took place in either medium. This accords well with the finding of WILBUR (1959) and KENWORTHY and CRABB (1963) that the gut of piglets is sterile at birth.

A graphical presentation of the development of the intestinal flora in young pigs is shown in Fig. 1. The graphs represent the counts of the main bacterial flora components at five different points of time (2, 6 and 18 hours and 1, 5 and 12 days of age) which we regarded as characteristic, and more conclusive than the combined illustration of the data of Table I.

of suckling piglets until 12 days age. (In logarithms of germ counts.)

| Jejunum | | | | | Ileum | | | | | Colon | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| L | S | C | Cl | Sp | L | S | C | Cl | Sp | L | S | C | Cl | Sp |
| 2.0 | 5.9 | 2.0 | — | — | 5.3 | 6.2 | 4.7 | 2.3 | — | 5.9 | 6.7 | 5.0 | 2.2 | — |
| 4.3 | 5.1 | 5.2 | 4.6 | 1.3 | 3.7 | 5.3 | 5.1 | 3.2 | 0.7 | 4.3 | 8.1 | 8.5 | 7.1 | 6.1 |
| 5.3 | 5.8 | 5.0 | 3.2 | 1.0 | 7.0 | 6.0 | 6.6 | 6.2 | 2.5 | 8.2 | 8.2 | 8.4 | 8.4 | 5.3 |
| 6.7 | 5.2 | 4.0 | 6.5 | 0 | 8.1 | 7.6 | 6.8 | 7.0 | 3.2 | 8.8 | 8.6 | 8.4 | 9.7 | 6.3 |
| 5.4 | 4.2 | 3.6 | 1.9 | 0 | 7.7 | 7.5 | 6.8 | 5.8 | 1.8 | 8.4 | 8.7 | 8.5 | 8.1 | 7.4 |
| 6.6 | 5.0 | 4.8 | 3.1 | 1.0 | 8.1 | 7.1 | 8.0 | 7.6 | 1.7 | 8.6 | 7.6 | 8.1 | 8.5 | 5.0 |
| 7.9 | 5.9 | 2.6 | 3.2 | 0.5 | 9.2 | 8.5 | 8.1 | 5.6 | 2.0 | 9.5 | 9.0 | 8.5 | 8.9 | 4.7 |
| 8.0 | 7.0 | 7.0 | 6.8 | 2.3 | 7.0 | 6.5 | 7.7 | 6.7 | 1.3 | 8.6 | 8.1 | 9.0 | 7.7 | 5.0 |
| 7.5 | 4.1 | — | 1.2 | 0.9 | 9.1 | 7.3 | 8.0 | 7.5 | 5.4 | 9.4 | 8.5 | 8.2 | 9.1 | 6.3 |
| 8.0 | 7.3 | 7.3 | 5.6 | 0.5 | 7.5 | 7.1 | 7.0 | 7.3 | 3.5 | 9.5 | 8.8 | 8.8 | 8.8 | 5.8 |
| 7.8 | 4.2 | 1.4 | 4.2 | 0.8 | 8.9 | 8.8 | 8.5 | 8.8 | 4.8 | 8.9 | 7.5 | 7.8 | 9.2 | 6.6 |

Fig. 2 shows the growth of streptococci in various isolation media. Two hours after birth, ubiquitous streptococci growing out on common blood agar, were found to predominate throughout the digestive tract over D-streptococci which grew exclusively on the selective Edwards agar (PESTI, 1962). However, six hours after birth the streptococci growing out on the two kinds of medium corresponded to one another in respect of count and colony morphology (enterococci).

Changes in, and relative proportions of, the vegetative and spore-bearing forms of clostridia were also examined. The graphical presentation of the respective mean counts, of logarithms determined until the 36th hour of age and subsequently until the 12th day, is shown in Fig. 3.

Haemolytic *E. coli* bacteria could never be isolated from the gut flora of healthy piglets, although the colonies growing out on blood agar plates were very carefully scrutinized.

## Discussion

Observations by others and by ourselves unequivocally indicate that the main characteristics of gut flora in suckling pigs are the absence of haemolytic *E. coli* bacteria and the quantitative relations of the main bacterial components. In healthy suckling pigs, the main components are lactobacilli in all intestinal segments distally from the stomach, followed in sequence by streptococci and coliforms as the third characteristic component, occurring in a much lower number than the other two. Although the counts of the three main com-

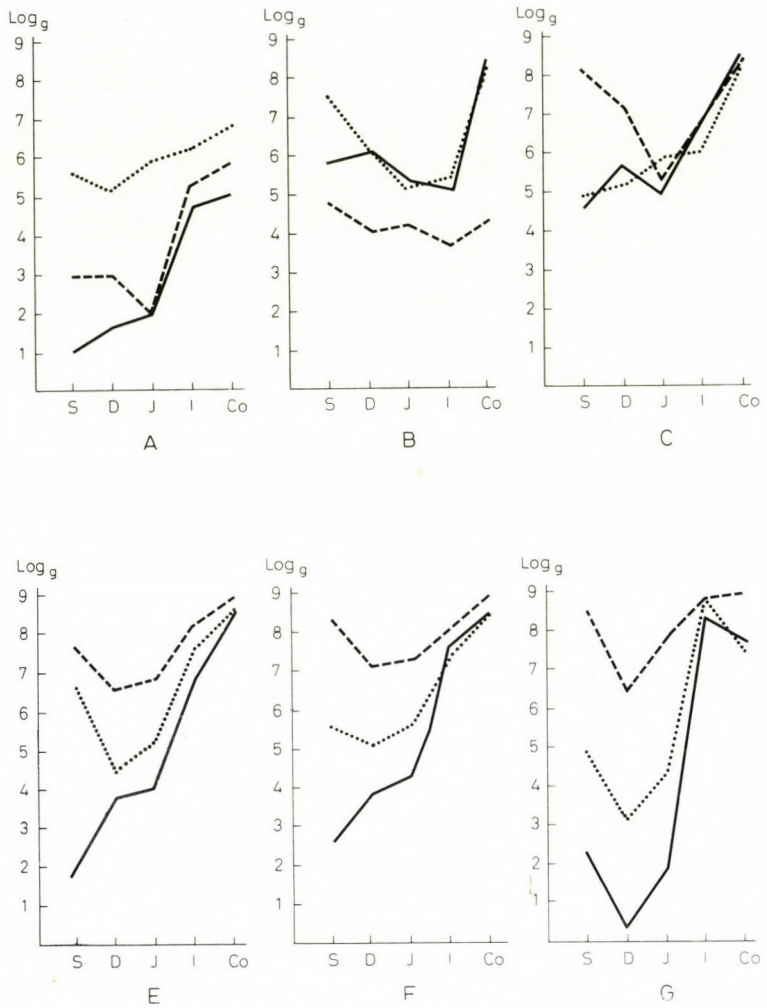


*Fig. 1.* Development of gastro-intestinal flora in suckling piglets. - - - - - lactobacilli; . . . . . D streptococci; ——— coliformes. S, stomach, D, duodenum; J, jejunum; I, ileum; Co, colon; A, 2-hour-old piglets; B, 6-hour-old piglets; C, 18-hour-old piglets; E, 1.5-day-old piglets; F, summarized data of 2—11 days old piglets; G, 12-day-old piglets

ponents varied characteristically with each segment, the lactobacillus—streptococcus—coliform ratio was practically constant throughout the digestive tract and, as shown in Table I and Fig. 1, it became established within about 1.5 days after birth and did not notably change until 12 days of age, *viz.*, until the conclusion of the experiment. Accordingly, as 1.5-day-old piglets already possess a practically "finished" gut flora, a follow-up of development of this flora requires the study of still younger animals.

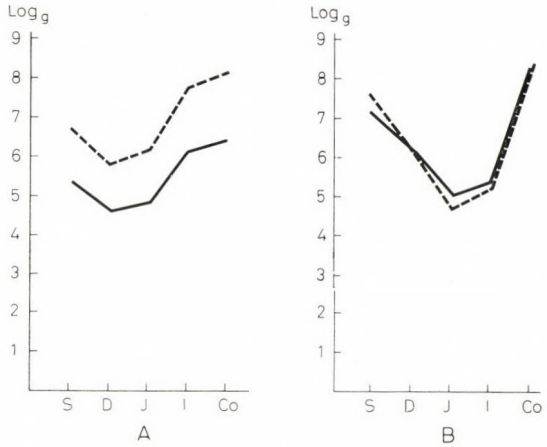


*Fig. 2.* Quantitative changes of streptococci in the alimentary tract of piglets. - - - - - ubiquitous streptococci; ——— D streptococci; A, 2-hour-old piglets; B, 6-hour-old piglets

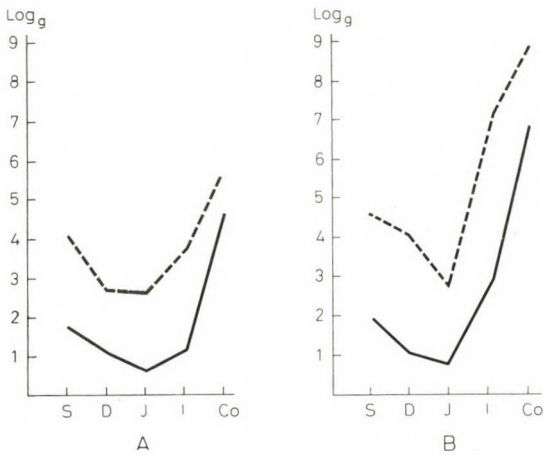


*Fig. 3.* Vegetative cells and spores of clostridia in the pig intestine. - - - - - - vegetative cells; ——— spores; A, summarized data until 1.5 days of age; B, summarized data between 1.5 and 12 days of age

As can be seen in Fig. 1, in 2-hour-old piglets, which had already sucked colostrum, the dominant gut flora component is represented by D streptococci. Lactobacilli and coliforms occur in almost equal amounts in those segments which are distally from the jejunum. At 6 and 18 hours after birth, the gut flora is still not fully balanced, but a quantitative predominance of lactobacilli can already be recognized in certain segments at 18 hours. Table I clearly shows that no such major fluctuations of gut flora are taking place between days 1, 5 and 12, except a definite reduction in coliforms in the duodenum at 12 days.

Fig. 2 shows the growth of streptococci in various media. Two hours

after birth, ubiquitous streptococci growing on blood agar were found to predominate over D-streptococci growing out exclusively on the selective Edwards medium, throughout the entire digestive tract. However, six hours after birth the streptococci growing out on the two kinds of medium corresponded to one another both in respect of counts and colony morphology.

Thus the gut flora of piglets killed 2, 6 or 18 hours after birth was characterized by a certain disbalance, which lasted until about 1.5 days of age. The components of the gut flora clearly originated from the environment of the animals, to judge from bacteriological studies of the teats of the sow, and litter and objects in the pen (NAGY, 1970). However, in the subsequent period up to the 36th hour after birth the quantitative relations of the gut flora changed markedly, independent of the environmental flora. After 1.5 days of life, the intestinal flora appeared to be controlled by inner principles. The quality and quantity of germs taken up by the animal orally from its immediate surroundings are not likely to change notably in a given environment, yet the gut flora develops towards the predominance of lactobacilli, which is characteristic of the healthy suckling pig.

This means that the composition of the healthy gut flora depends on an inner regulating mechanism which takes effect shortly after birth so that it is capable of establishing a state of balance within about 1.5 days of life.

It follows that untoward environmental influences on the gut flora are not so much the result of some "contamination", as of the deleterious effect of management failures on the operation of the inner regulating mechanism. This accords well with the previous finding (SINKOVICS, 1973) that environmental stessors affecting the organism of the animal as a whole elicited pathological alterations of gut flora under both laboratory and field conditions. On the other hand, as it is known from literary data and our own experience, certain haemolytic *E. coli* strains known as pathogenic for swine, did not durably establish themselves in the gut on oral administration and no symptoms were associated with their temporary presence therein.

Certain practical observations also weigh in favour of the above interpretation. It is known that the recurrent digestive troubles of neonatal pigs and weanlings could never be prevented by exaggerated disinfection if the management technology and the housing of the animals were inadequate.

The quantitative relationships of vegetative and spore-bearing forms of clostridia were practically the same a few hours after birth and days later. It was found earlier (SINKOVICS, 1972) that 20—35 days old healthy pigs had spores in the gut at low counts, but no vegetative forms of clostridia, suggesting the presence of a factor inhibiting clostridial growth in the small intestine. In the present studies on younger animals this phenomenon was less conspicuous. Markedly numerous vegetative clostridia and spores reached, across the gastric acid barrier, where they tended to decrease in number.

It may be concluded that the characteristic gut flora of the suckling pig becomes established by 1.5—2 days after birth. The details of the developmental process should be elucidated by an intensive follow-up of gut flora changes in still younger pigs.

## Summary

The development of gut bacterial flora was examined on healthy pigs killed at predetermined intervals from birth to 12 days of age. The characteristic gut flora of the healthy suckling pig had become established within 1.5—2 days after birth and did not notably change thereafter, until the end of the observation period at 12 days of age. Initially, the dominant component was formed by ubiquitous streptococci, whose count considerably surpassed that of D streptococci, but later it fell to a low level. The vegetative and spore-bearing forms of clostridia were present in all segments of the digestive tract from shortly after birth to the end of the observation period, but both forms tend to decrease in number in the small intestine.

It appears that intestinal flora development in the neonatal pig is to a certain degree independent of the quality and quantity of environmental flora, being controlled by an inner regulating mechanism, which acts towards the establishment of a balanced intraluminal biocoenosis.

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Address of the authors: Dr. György Sinkovics, 1078 Budapest, Landler J. u. 2; Dr. Balázs Juhász, 1042 Budapest, Kitaibel P. u. 4, Hungary.

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# ELEKTRONENMIKROSKOPISCHE UNTERSUCHUNGEN AN ENTWICKLUNGSSTADIEN VON *EIMERIA MAXIMA*

## IV. DIE FEINSTRUKTUR DER SCHIZONTEN UND MEROZOITEN*

Von

H. MEHLHORN**

Zoologisches Institut der Universität Bonn,
Abteilung für Protozoologie (Leitung: Prof. E. SCHOLTYSECK)

(Eingegangen am 25. Oktober 1973)

Keine Phase des Entwicklungszyklus von Coccidien weist so viele Modifikationen bei den einzelnen Arten auf wie die Schizogonie. So schien es lange Zeit nahezu unmöglich, eine Typisierung dieses ungeschlechtlichen Vermehrungsprozesses vorzunehmen.

Zu unterschiedlich erwiesen sich die Vorgänge z. B. bei *Eimeria bovis* (SHEFFIELD und HAMMOND, 1967), *E. intestinalis* (SNIGIREVSKAYA, 1969), *E. callospermophili* (ROBERTS u. Mitarb., 1970), *E. stiedae* (ČERNÁ und SENAUD, 1971) und den cystenbildenden Organismen *Toxoplasma gondii*, *Besnoitia jellisoni*, *Frenkelia* spec. und *Sarcocystis tenella*, bei denen lange Zeit als einzige Vermehrungsform die als Endodyogenie bezeichnete innere Zweiteilung bekannt war (VAN der ZYPEN und PIEKARSKI, 1967; SHEFFIELD, 1968; SENAUD, 1967; KEPKA und SCHOLTYSECK, 1970; MEHLHORN und SCHOLTYSECK, 1973). Mit dem Nachweis der Coccidiennatur von *T. gondii* und *S. tenella* (WORK und HUTCHISON, 1969; OVERDULVE, 1970; SHEFFIELD und MELTON, 1970; FRENKEL u. Mitarb., 1970; ROMMEL u. Mitarb., 1972) wurden noch weitere Schizogonievorgänge im Katzendarm beschrieben und z. T. als Endopolygenie bezeichnet. So existieren schließlich so deskriptive Termini wie innere, äußere Schizogonie, Cytomerenbildung, Endodyogenie und Endopolygenie. Sie alle sind Ausdruck der ungeheuren Plastizität der Parasiten bei der Anpassung an die spezifischen Bedingungen des jeweiligen Wirtsgewebes. Erst durch jüngste Untersuchungen von SCHOLTYSECK (1972, 1973) an *E. tenella* und *E. stiedae* ergaben sich deutliche Hinweise, daß die Endodyogenie als grundlegender Prozeß aller Schizogonievorgänge anzusprechen ist. Bei dieser Betrachtungsweise spielt es dann keine Rolle mehr, ob die Endodyogenie durch viele oder wenige vorgeschaltete Kernteilungen erweitert wird, ob die Merozoitenanlagen an der Oberfläche oder an Einfaltungen entstehen, oder ob gar durch Cytomerenbildung eine weitere Entwicklungsphase eingeschaltet ist.

Vor diesem Hintergrund soll nun die Merozoitendifferenzierung des wenig pathogenen Hühnercoccids *Eimeria maxima* näher analysiert werden. Diese Untersuchung schließt sich an vorausgegangene zur Gamogonie an (MEHLHORN, 1972a, b, c) und beendet die Analyse des Entwicklungszyklus im Haushuhn.

* Mit Unterstützung der Deutschen Forschungsgemeinschaft.
** Herrn Prof. E. SCHOLTYSECK danke ich für die Überlassung des Themas und seine stete Beratung.

## Material und Methode

Insgesamt 16 Hünchen (Weißes Leghorn) wurden im Alter von ca 3 Wochen mit 1—2 Millionen sporulierten Oocysten eines reinen *Eimeria maxima* Stammes oral infiziert. Am 1.—4. Tag p. i. wurden die Tiere dann getötet und Teile des Epithelzellenbereichs aus dem Duodenum entfernt. Die Weiterbehandlung dieser Gewebe für die elektronenmikroskopische Untersuchung erfolgte nach den gleichen Methoden, die schon in vorausgegangenen Publikationen beschrieben worden sind (MEHLHORN, 1972a, b, c). Ich möchte es an dieser Stelle nicht versäumen, Herrn Dr. A. Haberkorn von den Farbenfabriken BAYER AG für die Durchführung der Infektionen meinen Dank auszusprechen.

## Ergebnisse

### *Zur Feinstruktur der Schizonten*

Bei den hier analysierten Schizonten handelt es sich nach der Definition von MILLARD et al. (1972) um die »kleinen« Stadien, die nur etwa 8—16 Merozoiten produzieren, während die von ihnen beschriebene erste Schizontengeneration ja immerhin 25—50 Merozoiten ausdifferenzieren soll. Allerdings konnten bei den vorliegenden Untersuchungen nur zwei Schizogonien beobachtet werden. Die erste am zweiten Infektionstag, die zweite ca 72 Stunden post infectionem. Inwieweit sich jedoch vor diese beiden »kleinen« Schizontengenerationen noch eine weitere eingeschaltet hat, konnte mit der elektronenmikroskopischen Untersuchungstechnik bisher noch nicht festgestellt werden.

Nachdem die Merozoiten des zweiten Infektionstages in eine Wirtszelle eingedrungen sind, strecken sie sich stark in die Länge. Dabei werden die beiden inneren, miteinander verhafteten Pellikulamembranen zerrissen und schließlich zurückgebildet, so daß der Schizont, der die nächste Merozoitengeneration produziert, nur von einer Elementarmembran umschlossen ist (Abb. 1, 2a, 2c, 4). Die beobachteten Mikroporen behalten ihren typischen

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*b*, Längsschnitt durch einen Schizonten (SCH) mit 5 gleich weit differenzierten Merozoitenanlagen (EM). Zwei Merozoiten werden durch Einfaltungen von der Oberfläche her gebildet (IN). ×15 000

Verzeichnis der Abkürzungen: AM, Reservestoffgrana (Amylopectin); C, Conoid; DK, Dunkler Körper unterhalb des Conoids von Merozoitenanlagen (PO ?); EM, Merozoiten in der Entwicklungsphase; ER, Endoplasmatisches Retikulum; FW, Fingerförmiger Fortsatz der Wirtszelle; GO, Golgi-Apparat; I, Elektronendichter Einschluß in Mitochondrien; IM, Innere Membran der Merozoitenpellikula; IN, Invagination; L, Lipoid; LM, Begrenzungsmembran des Parasiten; LP, Begrenzungsmembran der parasitophoren Vakuole; MI, Mitochondrium; MIH, Mitochondrium der Wirtszelle; MM, Mittlere Membran der Wirtszelle; MP, Mikropore; N, Nukleus; NM, Begrenzungsmembranen des Nukleus; NU, Nukleolus; OF, Anschnitt des Ausführgangs der PO = Öffnung an der Conoidspitze; OM, Äußere Membran der Merozoitenpellikula; P, Polring; PG, Proteingrana; PO, Rhoptrien (Paariges Organell); PP, Hinterer Polring; PT, Zuspitzung des Nukleus (Spindelpol?); PV, Parasitophore Vakuole; R, Conoidale Ringe; RW, Ringwulst bei Merozoitenanlagen = späterer PP; SC, Der Schnitt erfolgte in dieser Ebene; SCH, Schizont; SMT, Subpellikuläre Mikrotubuli; WZ, Wirtszelle; WZK, Wirtszellkern

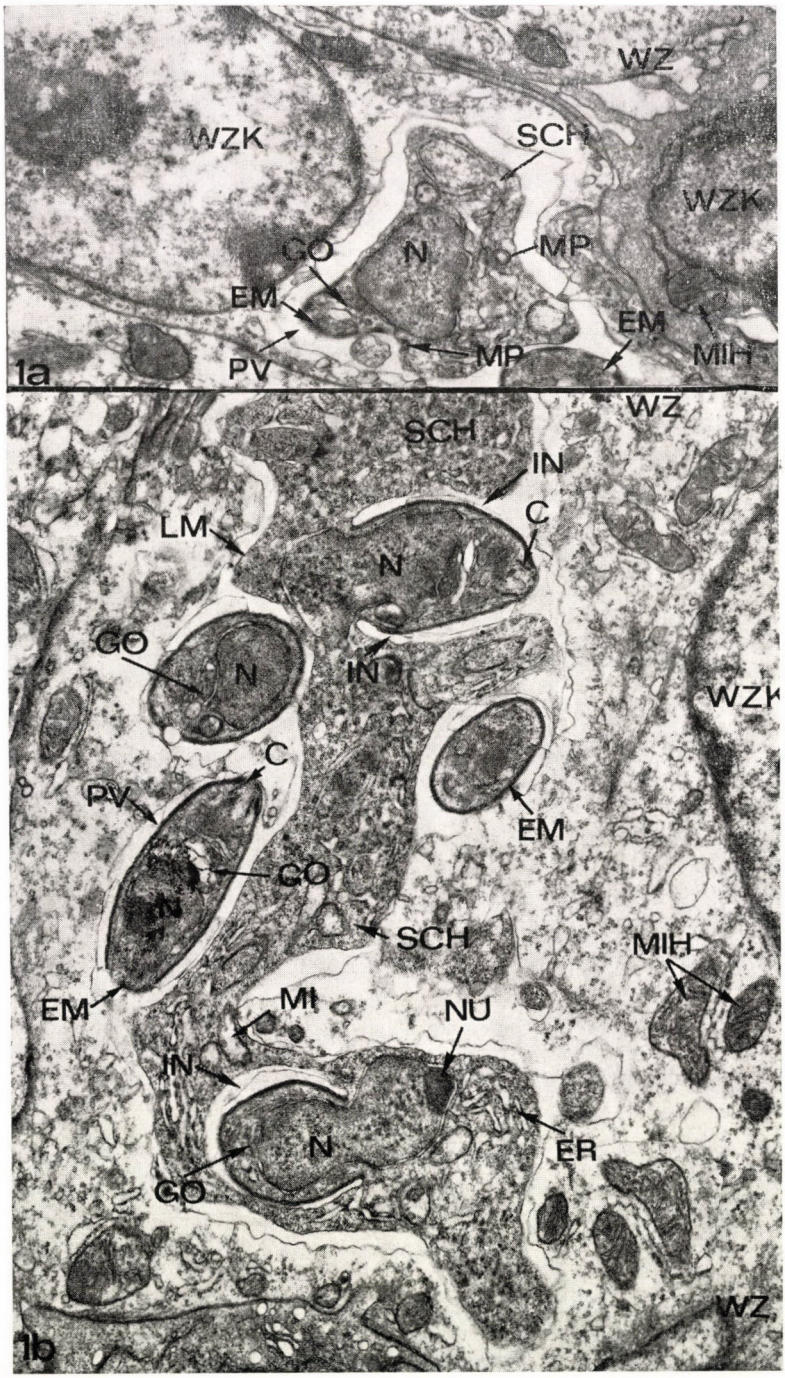


*Abb. 1a, b. Eimeria maxima.* Elektronenmikroskopische Aufnahmen. *a*, Querschnitt durch einen langgestreckten Schizonten (SCH), wobei zwei in der Entwicklung begriffene Merozoiten (EM) getroffen wurden. Das Wirtsgewebe zeigt keinerlei Schädigung (WZ). ×15 000.

Innenzylinder bei. An diesen Stellen bleiben also die beiden inneren Membranen als Relikte bewahrt (Abb. 1a, 4). Gleichzeitig treten die Mikroporen in größerer Anzahl als bei Merozoiten auf, wo sie auf die Einzahl beschränkt sind. Die kleinen Schizonten von *E. maxima* werden höchstens 20 $\mu$ groß und erreichen damit im Vergleich zu *E. tenella* (50—60 $\mu$; SCHOLTYSECK, 1973) recht bescheidene Abmessungen. Sofort zu Beginn des Wachstums teilt sich der Zellkern durch Zweiteilung in 2, 4, 8 und 16 Tochterkerne (Abb. 1a, 1b, 2a, 4). Während dieses Vorganges bleiben stets die Kernmembranen erhalten. Als maximale Anzahl von Tochterkernen wurden 16 ermittelt, die sich entlang der Oberfläche des langgestreckten und verzweigten Schizonten verteilen. Etwa gleichzeitig beginnt die Merozoitenbildung (Abb. 1b, 4), da auf Schnitten durch Schizonten meist gleich weit entwickelte Merozoiten angetroffen werden können. Offenbar setzt die Ausformung der Merozoiten schon vor der Beendigung der letzten Kernteilungsphase ein, so daß sich dann der Nukleus erst während der Merozoitenbildung teilt und sich somit an einem zweizipfligen Kern zwei kappenartige Merozoitenanlagen befinden (Abb. 2c).

Die Differenzierung der Merozoiten beginnt mit der Bildung von kappenartigen Vorstülpungen oberhalb der an der Peripherie des Schizonten befindlichen Nuklei (Abb. 2e, 2f). Dabei ist zu beobachten, daß unter der begrenzenden Elementarmembran die beiden inneren Membranen der späteren Merozoitenpellikula neu angelegt werden (Abb. 2e, 2f). Sie sparen am vorderen Pol eine kreisrunde Öffnung aus und sind dort zu einem Polring verdickt (Abb. 2e, 4). Der spätere hintere Polring ist auch schon vorhanden und stellt offenbar das Bildungszentrum der inneren Membranen dar, denn bei Schnitten durch Entwicklungsphasen der kegelförmigen Vorstülpungen kann stets dieser Ringwulst bemerkt werden (Abb. 2e, 4). Schon die jüngsten Vorstülpungen enthalten ein Conoid. Unmittelbar darunter liegt in allen Fällen ein kugelförmiges Organell, das von einer Elementarmembran begrenzt wird und das osmiophiles, granuläres Material enthält (Abb. 2e, 2f). Ähnliche Körper wurden schon von SHEFFIELD und HAMMOND (1967) bei sich entwickelnden Merozoiten von *E. bovis* beschrieben und als Vorläufer der paarigen Organelle diagnostiziert. Die Befunde an *E. maxima* weisen in die gleiche Richtung, denn in etwas älteren

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Die subpellikulären Mikrotubuli (SMT) beginnen in Kernnähe bündelartig (Pfeile) und ordnen sich dann fächerartig unter der Pellikula an. ×34 000. *c*, Schnitt durch einen Schizonten, bei dem noch während der letzten Kernteilung (= zweizipfliger Kern) die Merozoitenbildung einsetzt (EM). An einem der beiden durch die starken Pfeile gekennzeichneten »Kernzipfel« tritt eine Spitze (PT) in Erscheinung, die möglicherweise einen Spindelpol darstellt. ×25 500. *d*, Die subpellikulären Mikrotubuli werden in Kernnähe als Bündel (SMT) angelegt. ×48 600. *e*, Längsschnitt durch eine junge Merozoitenanlage (EM). Unterhalb des Conoids (C) befindet sich ein dunkler, von einer Elementarmembran begrenzter Körper (DK), der möglicherweise den Vorläufer der Rhoptrien darstellt. Das Conoid ist aus einzelnen Tubuli (Pfeil) aufgebaut. ×37 800. *f*, Parallelschnitt zu Abb. 2e. Der Doppelpfeil deutet auf die Elementarmembran des dunklen Körpers (DK), während der einfache Pfeil auf die Tubuli des schräg getroffenen Conoids (C) verweist. ×43 700

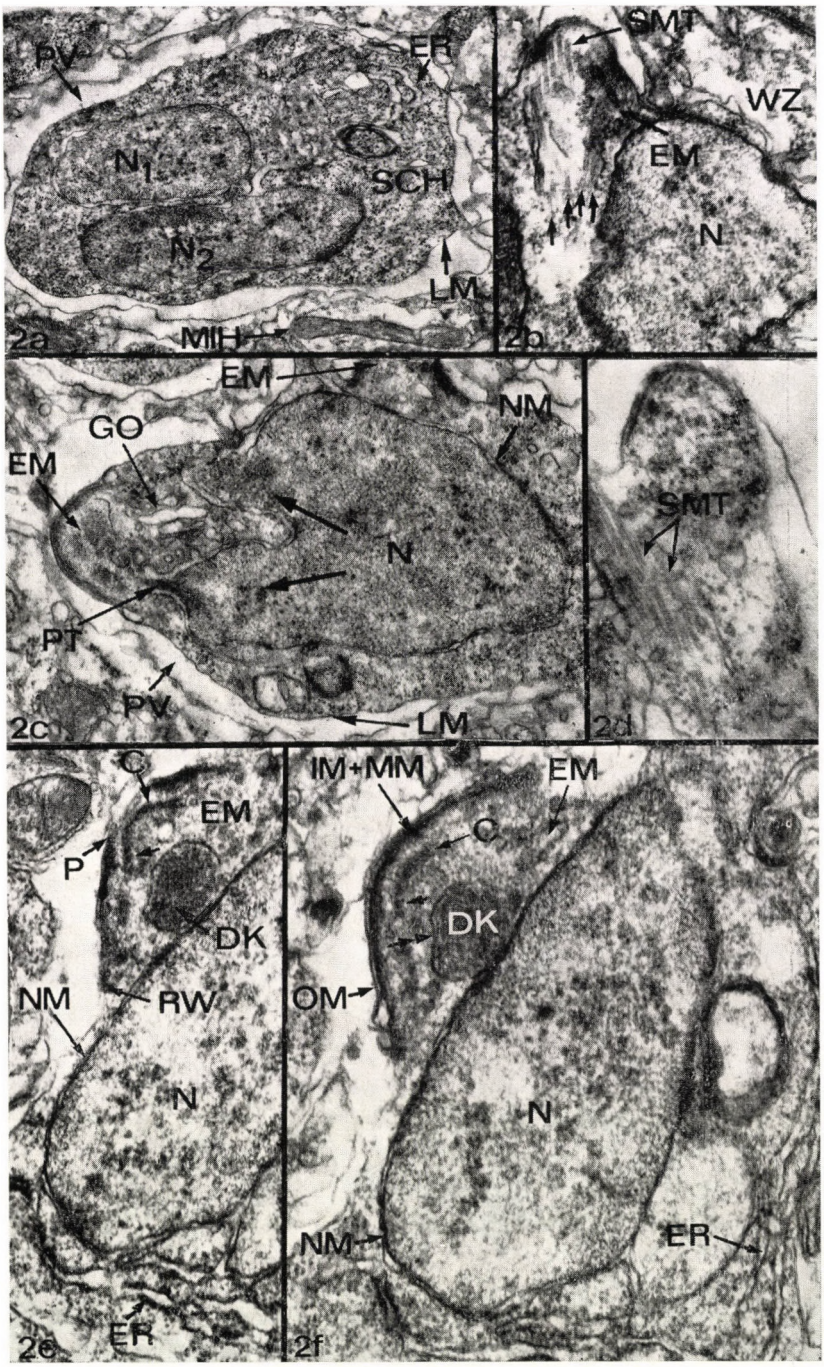


*Abb. 2a—f. Eimeria maxima.* Elektronenmikroskopische Aufnahmen. *a*, Schnitt durch einen Schizonten (SCH) mit zwei Kernen ($N_1$, $N_2$). Als Zellbegrenzung ist eine einzige Elementarmembran (LM) ausgebildet. ×18 000. *b*, Oberflächenschnitt durch eine Merozoitenanlage.

Entwicklungsstadien finden sich Anschnitte von zwei derartigen Strukturen, und noch später ziehen »Halsteile« zum apikalen Zellpol der entstehenden Merozoiten. Unmittelbar vor dem Nukleus des späteren Merozoiten findet sich schon zum frühesten Zeitpunkt ein gut ausgebildeter Golgiapparat (Abb. 1b). Die Mikroporen werden in Einzahl ebenfalls zu diesem Zeitpunkt angelegt. Sie deuten damit in ihrem Bau und in ihrer Ausformung auf eine genetische Determination, denn als Nahrungsaufnahmeorganell erscheinen sie bei sich entwickelnden Merozoiten verfrührt. Die subpellikulären Mikrotubuli werden als Bündel in Kernnähe angelegt und mit dem Wachstum der Vorstülpung gleichmäßig unter der Oberfläche des Merozoiten verteilt. Dabei entstehen bei Tangentialschnitten fächerförmige Aspekte (Abb. 2b, 2d, 5). Die Vorstülpung wird stetig größer, und zwar geschieht das dadurch, daß nach und nach immer mehr Cytoplasma des Schizonten von der basal wachsenden Kegelkappe umschlossen wird. Schließlich haben die Merozoiten schon fast ihre spätere Länge erreicht, sind aber noch mit dem Restschizonten verbunden (Abb. 3g). Durch diese Verbindungszone verlaufen in vielen Fällen Lakunen des Endoplasmatischen Retikulums (ER), die damit einen engen Kontakt der neugeformten Merozoiten mit dem Schizonten bis zur endgültigen Abschnürung gewährleisten. Im Restkörper konnten gelegentlich Membranstrukturen beobachtet werden, deren Funktion und Herkunft unbekannt blieb. So beginnen Membranen im Cytoplasma und verlaufen völlig geradlinig über ca 10 $\mu$. Im Abstand von etwa 0,5 $\mu$ befindet sich dann stets eine völlig identische Membranstruktur, die durch ihre parallele Anordnung dem Ganzen einen säulenartigen Charakter verleiht.

Die Ausbildung der Merozoiten erfolgt zwar stets an der Peripherie, jedoch gelegentlich nicht in Form einer Vorstülpung, sondern durch Einfaltung der begrenzenden Elementarmembran des Schizonten. Nur so sind nämlich die elektronenmikroskopischen Schnittaufnahmen zu erklären, auf denen sich die Merozoiten im »Innern« des Schizonten an einem Spaltraum befinden (Abb. 1b, 4). Die Frage nach dem Mechanismus dieser beiden Vorgänge und die Klärung der Faktoren, die solche Entwicklungsrichtungen steuern, bedürfen hier noch weiterer Untersuchungen.

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(OM) stellt eine Elementarmembran dar, während die beiden inneren Membranen als einheitliche, dicke Schicht erscheinen (IM + MM). ×144 000. *e*, Längsschnitt durch den apikalen Pol eines gerade eingedrungenen Merozoiten. Die Begrenzungsmembran der parasitophoren Vakuole ist nämlich noch nicht überall ausgebildet (LP). Der Kernspaltraum scheint an der Bildung der vorgelagerten Golgivesikel (GO) beteiligt zu sein (PFeil). ×21 600. *f*, Schnitt durch den apikalen Pol eines Merozoiten, dessen Conoid (C) vorgeschoben ist und dabei dem Polring (P) vorn aufsitzt (starke Pfeile). An der Spitze des Conoids befindet sich eine Öffnung (OF), die den verschmolzenen Halsteil der Rhoptrien (PO) darstellt. ×50 000. *g*, Schnitt durch den hinteren Pol eines noch mit dem Schizonten (SCH) verbundenen Merozoiten (EM). Das endoplasmatische Retikulum verläuft durch die »Brücke« (ER). ×30 000. *h*, Schnitt durch ein typisches tubuläres Mitochondrium (MI), das einen opaken Einschluß (I) enthält. ×18 000. *i*, Längsschnitt durch eine Mikropore im inaktiven Zustand (MP). ×90 000

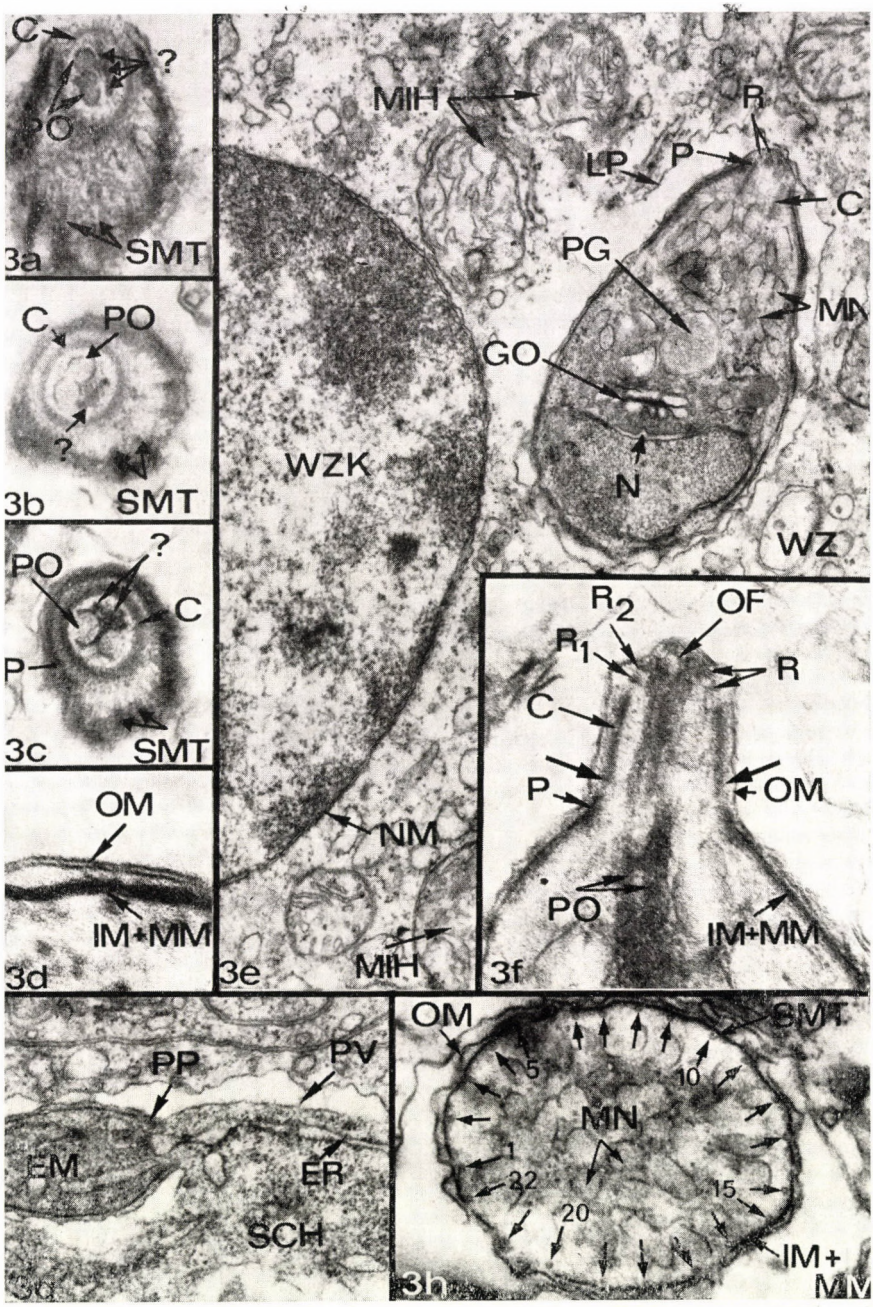


*Abb. 3a—i. Eimeria maxima.* Elektronenmikroskopische Aufnahmen. *a—c*, Querschnitt durch die unmittelbare Zellspitze eines Merozoiten. Innerhalb des Conoids (C) finden sich die Anschnitte der Rhoptrien (PO), daneben noch drei in einer Reihe angeordnete tubulöse Elemente (?). Die subpellikulären Mikrotubuli (SMT) verschmelzen am vorderen Pol mit dem Polring (P). *a, b, c* ×45 000. *d*, Schnitt durch die Merozoitenpellikula. Die äußere Schicht

### *Der Feinbau der Merozoiten*

Die Merozoiten aller hier beobachteten Generationen erscheinen gleichartig strukturiert. Sie sind langgestreckt und erreichen im differenzierten Zustand eine Länge von 7—9 $\mu$, während ihr Querschnitt dann etwa 1,8—2,5 $\mu$ mißt. Die Merozoiten werden von einer Pellikula umschlossen, die aus drei Schichten besteht. Die äußere umhüllt in Form einer Elementarmembran den gesamten Parasiten. Die beiden inneren stellen ebenfalls Elementarmembranen dar; sie sind aber eng aneinander geheftet, so daß sie als eine einheitliche, dicke Schicht erscheinen. Lediglich an einigen Stellen werden die beiden Schichten getrennt und somit überhaupt erst diagnostizierbar (Abb. 3d). Diese inneren Pellikulaschichten sparen am vorderen und hinteren Zellpol je eine kreisrunde Öffnung aus. Der Rand dieser Öffnung ist in beiden Fällen verdickt und formt so einen vorderen und hinteren Polring aus (Abb. 3c, 3e, 3f, 6, 7). Die Polringe dienen als Ansatzpunkt für subpellikuläre Mikrotubuli, die sich unter der inneren Pellikulaschicht von vorderen zum hinteren Zellpol erstrecken und durch Kontraktion für die Bewegungen der Merozoiten sorgen sollten (Abb. 3c). Bei *E. maxima* wurden 22 von diesen Mikrotubuli beobachtet, die einen Durchmesser von etwa 180—200 Å aufweisen. Sie sind in regelmäßigen Abständen unter der Pellikula angeordnet (Abb. 3h).

Durch Einfaltung der äußeren Pellikulaschicht bei gleichzeitiger Unterbrechung der beiden inneren Membranen an der Basis dieser säckchenartigen Struktur entstehen die für Coccidien typischen Mikroporen (Abb. 6). Bei *E. maxima* Merozoiten ist dieses Organell in seiner Lage ortsspezifisch determiniert, denn es findet sich stets in Einzahl auf Höhe des Nukleus. Im Tangentialschnitt erscheint es in Form von zwei konzentrischen Ringen. Der äußere Durchmesser beträgt etwa 190 m$\mu$, während der innere bei etwa 110 m$\mu$ liegt. Die Invaginationstiefe bewegte sich in den meisten Fällen um den Wert von 240 m$\mu$. Eine Abschnürung von Nahrungsvakuolen konnte in diesem Stadium nicht beobachtet werden (Abb. 6).

Dieser Prozeß sollte bei beginnendem Wachstum und damit verbundener Substanzzunahme einsetzen. Dann allerdings nimmt die Anzahl der Mikroporen sehr stark zu; offenbar ist dann die gesamte Oberfläche befähigt, derartige Strukturen entstehen zu lassen.

In der vom vorderen Polring umschlossenen Öffnung befindet sich ein als Conoid bezeichnetes Organell, das sich als typisch erwies für die Merozoiten und Sporozoiten aller Sporozoen mit Ausnahme der Haemosporidien, Babesien und Theilerien (Scholtyseck, Mehlhorn und Friedhoff, 1970). Es besteht aus einem konusartigem Hohlzylinder mit zwei vorgelagerten Ringen. Diese sind mit dem Hohlzylinder und untereinander durch fibrillöse Elemente verbunden, so daß bei der räumlichen Rekonstruktion eine baldachinartige Struktur entsteht, die an ihrer Spitze eine kreisrunde Öffnung ausspart.

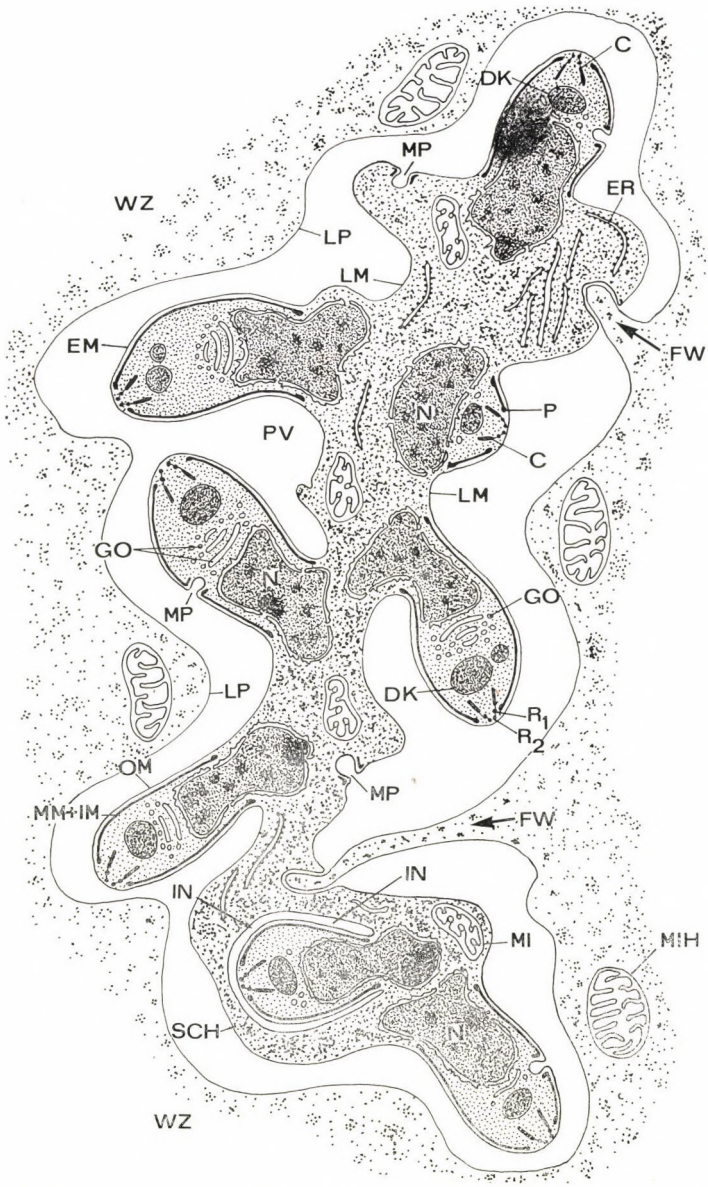


*Abb. 4. Eimeria maxima.* Schematische Darstellung eines langgestreckten Schizonten mit 8 Merozoitenanlagen (EM)

Diese Öffnung wurde von vielen Autoren als Apikalvesikel beschrieben. Sie stellt aber offenbar nicht anderes dar, als den Anschnitt der zu einer Einheit verschmolzenen Halsteile der Rhoptrien. Eine derartige enge Verbindung von Conoid und Rhoptrien erklärt den Sachverhalt, daß sich bei vorgestülptem Conoid die Ausführgänge der Rhoptrien immer noch an der unmittelbaren Zellspitze befinden (Abb. 3f, 6). Das Conoid kann also vorgeschoben werden, so daß der Merozoit dann mit einer warzenartigen Spitze erscheint, die eine mechanische Perforierung von Wirtszellmembranen ermöglicht. Dabei sitzt

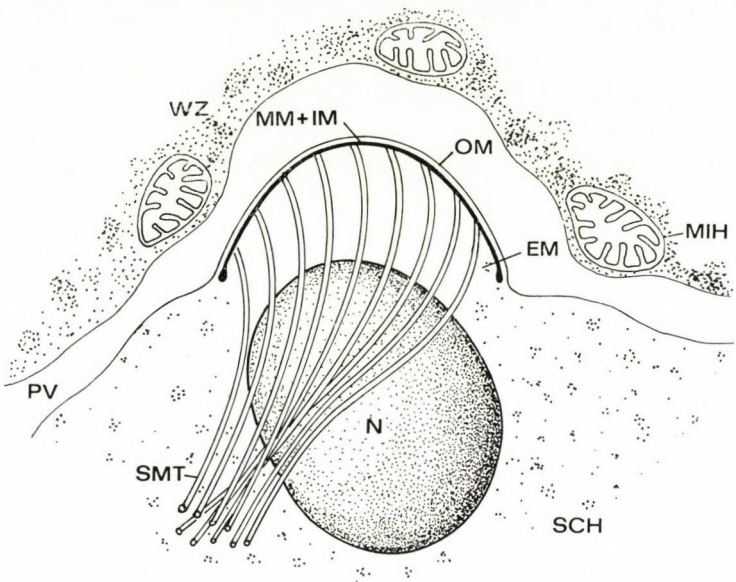


*Abb. 5. Eimeria maxima.* Schematische Darstellung der Anordnung der subpellikulären Mikrotubuli (SMT) bei jungen Merozoitenanlagen

das Conoid dem apikalen Polring vorn auf, die warzenartige Spitze kann also beim Auftreffen des Merozoiten auf die Wirtszellmembran nicht so leicht wieder eingedrückt werden (Abb. 3f). Die Wand des Hohlzylinders besteht aus einer Anzahl von Ring- bzw. Spiralelementen, von denen auf Längsschnitten meist 6—9 getroffen sind und die zur senkrechten Conoidachse einen Winkel von ca 60 Grad ausformen. In Durchmesser und Habitus erscheinen diese Elemente auf vielen elektronenmikroskopischen Aufnahmen als Mikrotubuli (Abb. 2e, 2f). Der konusartige Hohlzylinder hat an seiner Basis einen Durchmesser von etwa 0,34 $\mu$, der sich zur Spitze hin auf 0,28 $\mu$ verringert. Die Gesamthöhe der Conoids beträgt etwa 0,36—0,38 $\mu$. Die vielfach als Vesikel beschriebene Struktur am apikalen Merozoitenpol, die sich als Querschnitt durch die zu einer Einheit verschmolzenen Halsteile der Rhoptrien erklärt, mißt bei *E. maxima* 80—100 m$\mu$ im Durchmesser (Abb. 3f, 6).

Die Rhoptrien (paarige Organelle) verlaufen als gewundene, keulenförmige Strukturen etwa vom Ende des ersten Zelldrittels durchs Conoid zur unmittelbaren Zellspitze. Gelegentlich konnten auch die knollenartigen Enden in unmittelbarer Kernnähe beobachtet werden. Die Rhoptrien haben im Conoid einen Durchmesser von etwa 700 Å, erweitern sich dann auf ca 1300 Å und erreichen an ihren breitesten Stellen ca 2000 Å. Diese bei *E. maxima* paarigen Strukturen sind von einer Elementarmembran begrenzt, die allerdings wegen der starken Osmiophilie der Inhaltsstoffe meist nicht erkennbar ist. Lediglich in Fällen, wo das Lumen der Rhoptrien sehr elektronenlicht erscheint, ist dieser Grundaufbau sichtbar. Möglicherweise ist derartigen Aspekten ein Auspressen der osmiophilen Inhaltsstoffe vorausgegangen, denn »leere« Rhoptrien finden sich vorwiegend in Merozoiten, die einzeln in Wirtszellen liegen, also offenbar dort gerade eingedrungen sind.

In enger räumlicher Beziehung zu den Rhoptrien steht eine Vielzahl von Mikronemen, deren Windungsgrad bedeutend stärker ist als der der Rhoptrien (Abb. 3e). Sie werden ebenfalls von einer Elementarmembran begrenzt und erscheinen teilweise hohl. Sie messen ca 700 Å im Durchmesser, was den Halsteilen der Rhoptrien entspricht. Bei Querschnitten durch das Conoid können im Inneren drei nebeneinanderliegende Anschnitte von tubulösen Elementen beobachtet werden, deren Durchmesser bei etwa 240 Å liegt. Möglicherweise stellen diese Elemente Verbindungen der Mikronemen zu den Ausführgängen der Rhoptrien dar (Abb. 3a—c). Die Mikronemen erstrecken sich bis in die Kernregion, sind dort aber in ihrer Anzahl beschränkter als unmittelbar am apikalen Pol, wo sie den gesamten Zellinnenraum erfüllen.

Anschließend an die Mikronemenregion finden sich bis zu 9 kugelige Körper, die — von einer Elementarmembran umschlossen — granuläres Material geringerer Osmiophilie enthalten. Sie zeigen Durchmesser von etwa 3000—4000 Å und finden sich nur in ausgewachsenen Merozoiten (Abb. 3e, 6). Bei diesen Einschlüssen handelt es sich wahrscheinlich um die von anderen Merozoiten her bekannten Proteingrana (Cheissin und Snigirevskaya, 1965; Scholtyseck und Piekarski, 1965). Der Zellkern liegt durchweg im mittleren Zellabschnitt des Merozoiten. Er hat meist ovoid-kugelige Gestalt, kann aber bei diesen stark motilen Zellen auch recht unregelmäßig ausgebildet sein. In allen Fällen jedoch ist am Vorderende des Nukleus eine mehr oder minder tiefe Einbuchtung zu beobachten, in der die Zisternen und Vesikel des Golgi-Apparates angeordnet sind (Abb. 3e, 6). Es bestehen Anzeichen, daß der perinukleäre Raum an der Bildung der Golgivesikel beteiligt ist. In der charakteristischen doppelten Kernmembran befinden sich zahlreiche Kernporen, die eine enge Verbindung zwischen dem Karyoplasma und der übrigen Zelle ermöglichen. Der perinukleäre Raum ist bei den Kernen der Merozoiten gut entwickelt und er steht mit den Lamellensystemen des Endoplasmatischen Retikulums (ER) in Verbindung. Die Grundsubstanz des Karyoplasmas er-

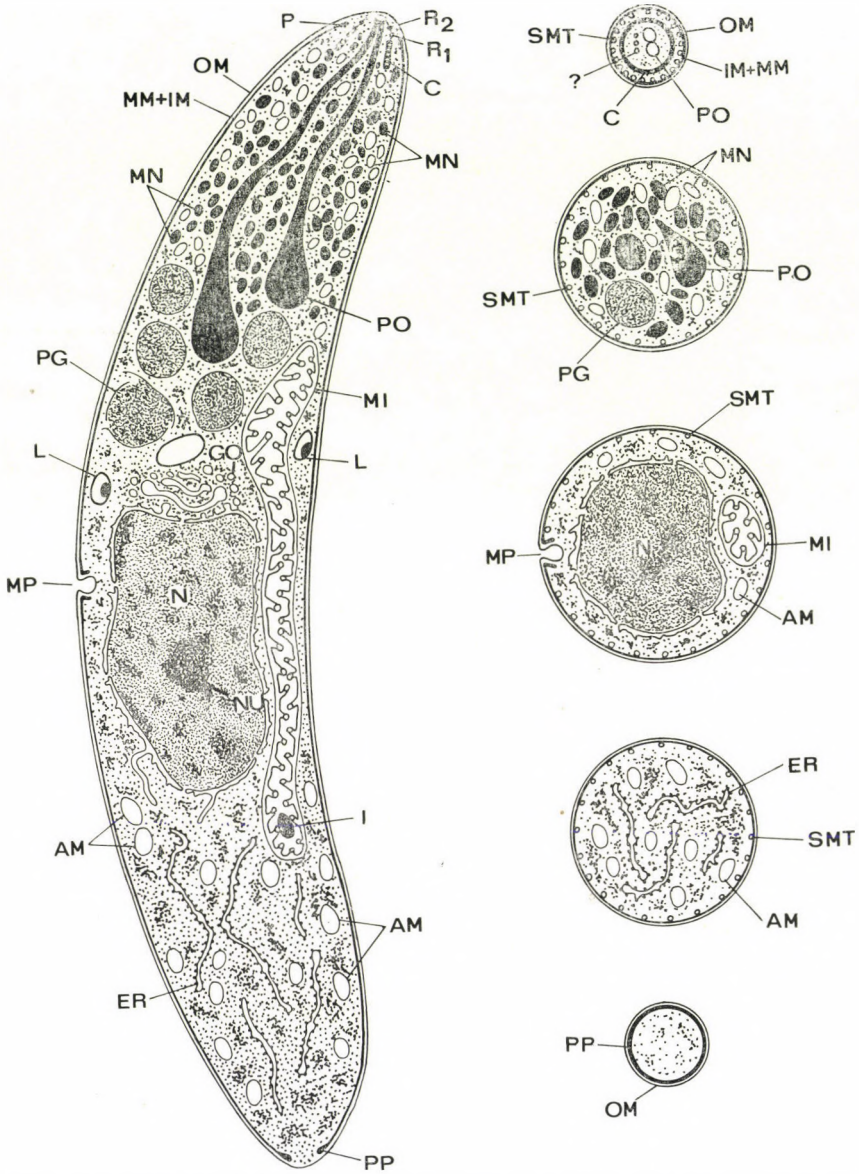


*Abb. 6. Eimeria maxima.* Schematische Darstellung eines Merozoiten im Längs- wie auch in Querschnitten

scheint nicht einheitlich, sondern sie ist durch Verdichtungen an einigen Stellen gekennzeichnet. Eine dieser Verdichtungen erweist sich als besonders groß und formbeständig und dürfte somit als Nukleolus angesprochen werden (Abb. 1b).

Der hintere Zellabschnitt des Merozoiten enthält eine Vielzahl von Lamellen der ER, die stellenweise blasenartige Aufschwellungen zeigen und

dadurch wie Perlschnuren wirken (Abb. 3g, 6). Bei noch nicht abgeschnürten Merozoiten stehen diese Lamellensysteme mit denen des Restkörpers in Verbindung. Entlang des ER und vornehmlich im hinteren Zellabschnitt befinden sich Reservestoffgrana, die nach der hier verwendeten Technik leuchtend weiß erscheinen. Diese Polysaccharidgrana sind in ihrer Anzahl beschränkt. Sie werden bei Merozoiten in weit geringerem Maße ausgebildet als etwa bei den Makrogameten. Lipoide liegen vereinzelt in Nähe des apikalen Kernpoles.

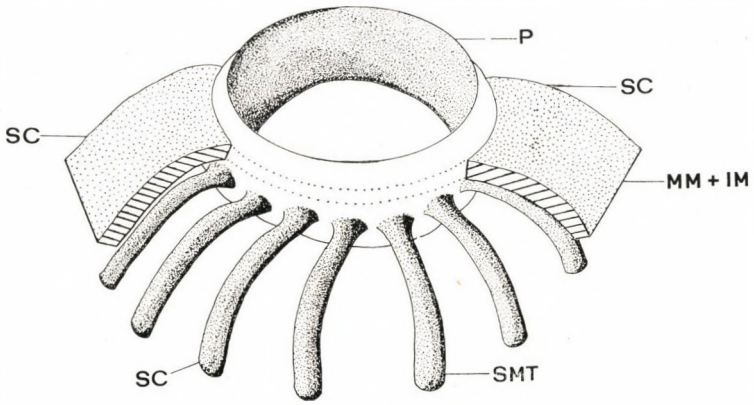


*Abb. 7. Eimeria maxima.* Schematische Darstellung der Verbindung von subpellikulären Mikrotubuli (SMT), inneren Pellikulamembranen (MM + IM) und Polring (P) am apikalen Pol eines Merozoiten. An den mit SC bezeichneten Stellen erfolgte aus zeichentechnischen Gründen eine Beschneidung der bezeichneten Strukturen

Die Merozoiten von *E. maxima*, die schematisch in Abb. 6 dargestellt sind, besitzen ein großes, langgestrecktes Mitochondrium mit tubulären Innenstrukturen. Es verläuft entlang des Nukleus und endet vorn etwa in der Region der Endkeulen der Rhoptrien. Auf einigen elektronenoptischen Aufnahmen schien es, als würde sich das Mitochondrium vor dem Kern gabeln. Es war jedoch nicht zu entscheiden, ob bei allen Merozoiten ein derartig charakteristischer Habitus in Erscheinung tritt. Opake Einschlüsse unbekannter Provenienz und Funktion konnten in den Mitochondrien der Merozoiten ebenso aufgefunden werden wie in denen der Makrogameten und Mikrogamonten (Abb. 6).

## Diskussion

### *Schizogonie*

In Anbetracht der jüngsten Ergebnisse bei Coccidien einschließlich der cystenbildenden Formen *Toxoplasma gondii*, *Sarcocystis tenella*, *Besnoitia jellisoni* und *Frenkelia* spec. (= M-Organismus) schien es nahezu unmöglich, eine Typisierung des als Schizogonie bezeichneten ungeschlechtlichen Ver-

mehrungsprozesses vorzunehmen, erwiesen sich doch die Vorgänge bei allen Arten in spezifischer Weise modifiziert. Auch konnten bei einigen Arten, wie z. B. *T. gondii*, verschiedene Bildungsmechanismen nebeneinander beobachtet werden.

Für die Gruppe der Eimerien galt lange eine Merozoitenbildung als typisch, bei der an der Schizontenoberfläche kappenartige Vorwölbungen auftraten, die dann schließlich zu den Merozoiten auswuchsen. Ein derartiger Prozeß wurde für *E. callospermophili*, *E. bovis*, *E. magna*, *E. falciformis* und *Isospora* spec. beschrieben (SHEFFIELD und HAMMOND, 1967; SCHMIDT u. Mitarb., 1967; SCHOLTYSECK u. Mitarb., 1968; SENAUD und ČERNÁ, 1968; PELLÉRDY u. Mitarb., 1971; MEHLHORN u. Mitarb., 1972). Bei *Eimeria intestinalis*, *E. nieschulzi*, *E. pragensis* und bei den sog. kleinen Schizonten von *E. stiedae* wurde gezeigt, daß die Merozoiten nicht durch Vorwölbungen entstehen, sondern durch Einfaltung der als Elementarmembran ausgebildeten Schizontenbegrenzung (SENAUD und ČERNÁ, 1968; COLLEY, 1968; SNIGIREVSKAYA, 1969; HELLER und SCHOLTYSECK, 1971). Bei *E. maxima* treten beide Bildungsmechanismen am selben Schizonten hervor. Daraus kann geschlossen werden, daß es sich nicht um prinzipiell unterschiedliche Vorgänge handelt, die eine Einordnung in verschiedene Typen rechtfertigen würde.

Die Endodyogenie (= innere Zweiteilung) war lange Zeit als einzige Vermehrungsweise der als Toxoplasmatea bezeichneten Gruppe von cystenbildenden Parasiten bekannt (*T. gondii*, *S. tenella*, *B. jellisoni* und *Frenkelia* spec. SHEFFIELD, 1966; VAN DER ZYPEN und PIEKARSKI, 1967; SENAUD, 1967; KEPKA und SCHOLTYSECK, 1970; SCHOLTYSECK u. Mitarb., 1970a; MEHLHORN und SCHOLTYSECK, 1973). Mit der Entdeckung der Katze als spezifischer Wirt von *T. gondii* konnte aber noch eine spezielle Form der ungeschlechtlichen Vermehrung nachgewiesen werden (COLLEY und ZAMAN, 1970a; SHEFFIELD, 1970; PIEKARSKI u. Mitarb., 1971). PIEKARSKI und Mitarbeiter (1971) bezeichneten diesen Vorgang als Endopolygenie, da dort mehrere Tochterzellen in einer Mutterzelle entstehen, an Stelle von nur zwei bei der Endodyogenie. Ähnlich der Endopolygenie verläuft die erste Schizogonie bei *E. callospermophili* in Zellkulturen (ROBERTS u. Mitarb., 1970). Dort beginnt die Merozoitenbildung ebenfalls im Innern des motilen Stadiums (Sporozoit) mit Kernteilungen, wobei während dieser Vorgänge schon die kappenartigen Anlagen der Merozoiten zu beobachten sind. Im Gegensatz zu *T. gondii* wölben sich diese Anlagen aber nach etwa 1/3 der Entwicklungszeit aus der Schizontenoberfläche hervor und gleichen dann dem von vielen Eimerien her bekannten Habitus.

Entsprechendes wurde bei *E. stiedae* und *E. magna* beobachtet, wo schon im Innern des Merozoiten die Tochterzellbildung beginnt (ČERNÁ und SENAUD, 1971; DANFORTH und HAMMOND, 1972).

Die Ergebnisse von SCHOLTYSECK (1972, 1973) an *E. tenella* und *E. stiedae* beweisen schließlich eindeutig den grundlegenden Zusammenhang zwischen

Endodyogenie und den verschiedenen Phänomenen der Schizogonie. Es konnte nämlich gezeigt werden, daß die Merozoitenbildung (= letzte Phase der Schizogonie) in Form der Endodyogenie verläuft, da sich der Schizont vorher in viele einkernige Cytomeren aufgegliedert hat. Somit ist die Schizogonie als eine sekundär durch Kernteilungen erweiterte Endodyogenie zu betrachten. Für *E. maxima* wie auch für *E. intestinalis*, *E. falciformis*, *E. stiedae*, *E. magna* und *Haemoproteus metchnikovi* (SNIGIREVSKAYA, 1969; ČERNÁ und SENAUD, 1971; DANFORTH und HAMMOND, 1972; STERLING und DE GIUSTI, 1972; MEHLHORN, SENAUD und SCHOLTYSECK, 1973) ergaben sich Anhaltspunkte, daß sich die Merozoiten ebenfalls schon während der letzten Kernteilung entwickeln. Im Gegensatz zu *E. tenella* und *E. stiedae* war allerdings keine Cytomerenbildung zu beobachten. Die Frage, ob das Phänomen zweier Merozoitenanlagen pro Kern genetisch verankert ist oder ob es auf Hemmungen im Kernteilungsrhytmus zurückzuführen ist, muß vorerst ungeklärt bleiben. Desgleichen die Frage, warum einige Arten wie *E. maxima* nur 8—16 Merozoiten pro Schizont ausbilden, andere dagegen bis zu 120 000 *(E. bovis)*. Zudem ist noch zu wenig bekannt über die Einflüsse des Wirtsgewebes auf diese Phase des Entwicklungszyklus.

Bei der Merozoitenbildung werden die subpellikulären Mikrotubuli zuerst als Bündel in Kernnähe angelegt und erst bei stetiger Vorwölbung der Merozoitenanlage symmetrisch unter der Pellikula verteilt. Ein derartiger Vorgang wurde zuvor auch schon bei *E. stiedae* beobachtet (HELLER und SCHOLTYSECK, 1971). Möglicherweise ist die Bildung dieser Mikrotubulibündel von Centriolen induziert, die bei der Kernteilung sich ja stets in räumlicher Nähe befinden (SCHOLTYSECK, 1973; MEHLHORN, SENAUD und SCHOLTYSECK, 1972; SCHOLTYSECK, MEHLHORN und SENAUD, 1972).

### *Merozoiten*

Die Merozoiten von *E. maxima* sind schlank-langgestreckt und entsprechen mit diesem Habitus und ihren Abmessungen den Verhältnissen bei vielen der bisher untersuchten Eimeriina, sowie *Toxoplasma gondii*, *Besnoitia jellisoni* und *Frenkelia* spec. (Lit. s. SCHOLTYSECK u. Mitarb., 1970a; PORCHET-HENNERE und VIVIER, 1971; SCHOLTYSECK u. Mitarb., 1973b). Bei *Sarcocystis tenella*, einem weiteren cystenbildenden Coccid, bei *E. falciformis* und *E. contorta* treten allerdings weit größere Merozoiten in Erscheinung, die 16 $\mu$ *(Sarcocystis)*, 25 $\mu$ *(E. falciformis)* und sogar 30 $\mu$ *(E. contorta)* erreichen können (SENAUD, 1967; HABERKORN, 1971; MEHLHORN und SCHOLTYSECK, 1973; MEHLHORN u. Mitarb., 1973). Inwieweit sich derartige Größenvariationen auf die spezifischen Bedingungen des jeweiligen Wirtsgewebes zurückführen lassen, bedarf noch der weiteren Klärung.

Unterhalb der charakteristischen Coccidienpellikula liegen bei *E. maxima* 22 subpellikuläre Mikrotubuli. Die Anzahl entspricht den Verhältnissen bei *E. necatrix*, *E. bovis*, *E. alabamensis*, *E. callospermophili*, *Isospora* spec., *Haemoproteus columbae* und bei den vier cystenbildenden Coccidien *(T. gondii; S. tenella; F.* spec.; *B. jellisoni)*. Eine ausführliche Literaturübersicht bietet die Arbeit: SCHOLTYSECK, MEHLHORN und SENAUD (1972). In dieser Arbeit konnten desweiteren Hinweise dafür gesammelt werden, daß die subpellikulären Mikrotubuli ursächlich in den Bewegungsablauf der Merozoiten integriert sind Dafür spricht auch, daß sie nur in motilen Stadien in Erscheinung treten und sofort abgebaut werden, sobald eine stationäre Phase (Schizont, Gamont) eingeleitet wird. Die Anzahl der Tubuli scheint artspezifisch determiniert zu sein. Sie könnte demnach als ultrastrukturelles Identifikationsmerkmal herangezogen werden.

In die Polring umschlossene Öffnung der beiden inneren Pellikulamembranen erstreckt sich bei Merozoiten und Sporozoiten aller Telosporidien mit Ausnahme der Plasmodien, Babesien und Theilerien ein als Conoid bezeichnetes Gebilde. Es besteht aus einem konusartigen Hohlkörper, dessen Wandung aus einer Anzahl von Spiralelementen aufgebaut ist, und zwei vorgelagerten Ringen, die untereinander und mit dem Konus durch fibrillöse Elemente verbunden sind. Auf Längsschnitten konnten hier nur 6—9 Anschnitte von solchen spiraligen Tubuli gefunden werden.

Die exakte Anzahl der Tubuli, aus denen das Conoid aufgebaut ist, kann zur Zeit nicht ermittelt werden, da sich das zur Verfügung stehende Methodenspektrum und gerade rechnerische Rekonstruktionen als unbefriedigend erwiesen haben (VIVIER, 1970). Eng verbunden mit dem Conoid sind die Ausführgänge der Rhoptrien (= Paariges Organell), die an der Spitze des Conoids zu einer Einheit verschmelzen. Querschnitte durch diese Struktur wurden sehr häufig als Vesikel angesehen. Die Verbindung von Conoid und Rhoptrien zu einer funktionalen Einheit erklärt die elektronenmikroskopischen Aufnahmen, auf denen das Conoid vorgeschoben ist und die Mündung der Rhoptrien sich trotzdem an der unmittelbaren Zellspitze befindet. Interessant ist der Befund an *E. maxima*, daß das Conoid im Fall der Vorstülpung schließlich vorn dem Polring aufsitzt. Damit scheint bewiesen, daß das Conoid seine Funktion in der Versteifung einer warzenartigen Vorstülpung erfüllt, mit deren Hilfe die Wirtszellmembran perforiert werden kann. Unterstützt wird dieser Vorgang offenbar noch durch Inhaltsstoffe der Rhoptrien, die ja in enger Kopplung mit dem Conoid stehen. Zur Natur etwaiger Inhaltsstoffe lassen sich zur Zeit allerdings noch keine definitiven Aussagen treffen (SENAUD, MEHLHORN und SCHOLTYSECK, 1972). In Anbetracht dieser Organellverbindungen am apikalen Pol von Merozoiten und Sporozoiten erscheint der von LEVINE (1970) vorgeschlagene Name »Apicomplexa« zur Kennzeichnung dieser gesamten Parasitengruppe (Sporozoa) erst im rechten Licht.

Plasmoiden, Babesien, Theilerien und das noch undefinierte Coccid *Myriosporides amphiglenae* besitzen kein Conoid, das selbst bei Gregarinen nachgewiesen werden konnte (SCHREVEL, 1968; DESPORTES, 1969; VÁVRA und MCLAUGHLIN, 1970; SHEFFIELD u. Mitarb, 1971; ORMIERES, 1972). Möglicherweise liegt das an dem Modus, wie eine Wirtszelle befallen wird, denn die Blutparasiten perforieren die begrenzende Wirtszellmembran nicht (LADDA u. Mitarb., 1969).

*E. maxima* besitzt wie alle bisher untersuchten Eimeria-Arten und *Isospora* spec. 2 Rhoptrien, die sich stets von einer Elementarmembran begrenzt erwiesen. Bedeutsam scheint der Befund, daß die Halsteile der Rhoptrien an der unmittelbaren Zellspitze zu einem gemeinsamen Stück verschmelzen, das dann auf Querschnitten als vesikelartige Struktur erscheint. Dieses gemeinsame Stück der Rhoptrien muß sehr kurz sein, denn noch innerhalb des Conoids verlaufen die Halsteile der Rhoptrien völlig getrennt, wie sich aus zahlreichen Querschnitten durch diese Region ermitteln läßt. Der obere, also gemeinsame Rhoptrienabschnitt steht durch fibrillöse Elemente mit dem oberen conoidalen Ring in Verbindung, so daß Conoid und Rhoptrien einen Funktionskomplex darstellen, der in identischer Form auch dann erhalten bleibt, wenn das Conoid vorgeschoben wird. Somit stimmt die Öffnung im »Baldachin« des Conoids mit dem Querschnitt durch den obersten Rhoptrienabschnitt überein.

Die Mikronomen der *E. maxima* Merozoiten entsprechen in Abmessungen, Morphologie und Anzahl den Verhältnissen bei anderen *Eimeria*-Arten und bei *Isospora* spec. (Lit. s. SCHOLTYSECK und MEHLHORN, 1970; SCHOLTYSECK u. Mitarb., 1973). Auf Querschnitten durchs Conoid finden sich bei *E. maxima* stets drei tubuläre Elemente, deren Durchmesser sich etwa auf 240 Å beläuft und die stets in einer Reihe angeordnet sind. Eine Funktion konnte ihnen bisher nicht zugeordnet werden. DUBREMETZ (1971) beobachtete bei *E. necatrix* auf Längsschnitten durchs Conoid zwei solche Strukturen von entsprechenden Durchmesser, desgleichen PORCHET-HENNERE und RICHARD (1970) bei *Aggregata eberthi*. Die Merozoiten von *E. tenella*, *E. nieschulzi*, *E. bovis*, *E. perforans*, *E. stiedae*, *E. miyairii*, sowie die »Endodyocyten« von *T. gondii*, *S. tenella*, *E. jellisoni* und *Frenkelia* spec. weisen diese Strukturen ebenfalls auf, wie aus den veröffentlichten Querschnittsbildern hervorgeht (SCHOLTYSECK und PIEKARSKI, 1965; SHEFFIELD und HAMMOND, 1966; SHEFFIELD, 1966; SENAUD, 1967; COLLEY, 1968; ANDREASSEN und BEHNKE, 1968; SHEFFIELD und MELTON, 1968; SENAUD und ČERNÁ, 1969; SCHOLTYSECK, KEPKA und PIEKARSKI, 1970). Aus den Aufnahmen von *E. maxima* ergibt sich jedoch, daß die Halsteile der Rhoptrien noch zusätzlich in dieser Schnittregion anzutreffen sind. Herkunft und Funktion dieser drei Tubuli sind noch völlig unbekannt und bedürfen der weiteren Klärung, wenn allerdings einiges dafür spricht, daß es sich um Verbindungen der Mikronemen mit den Rhoptrien handeln könnte.

Die Mikropore der Merozoiten von *E. maxima* befindet sich stets auf Kernniveau und entspricht in Aufbau und Größe dem für Sporozoen charakteristischen Typus (Lit. s. SCHOLTYSECK und MEHLHORN, 1970). Interessant ist, daß bei einigen Arten stets ein mehr oder minder spezifischer Platz der Pellikula zur Mikropore umgeformt wird. Offenbar ist die Anlage der Mikropore in irgendeiner Weise determiniert, denn schon in allerjüngsten, sich gerade entwickelnden Merozoiten kann dieses Nahrungsaufnahmeorganell beobachtet werden, obwohl es hier noch nicht benötigt wird. Ebenfalls in Nähe des Zellkerns sind bis zu 9 Proteingrana zu beobachten (CHEISSIN und SNIGIREVSKAYA 1965). Sie sind typisch für die Merozoiten der Gattungen Eimeria und Isospora, wurden jedoch bisher nicht oder zumindest nicht in gleicher Weise für *T. gondii*, *B. jellisoni*, *S. tenella*, *F.* spec. oder gar für die Merozoiten der Haemosporidien und Piroplasmen beschrieben (SCHOLTYSECK und MEHLHORN, 1973).

## Zusammenfassung

Die Schizogonie des Hühnercoccids *Eimeria maxima* wurde erstmals elektronenoptisch untersucht und mit entsprechenden Vorgängen bei anderen Sporozoen verglichen. Dabei ergab sich, daß sich vermutlich die hier zu Grunde liegenden Entwicklungsprozesse trotz aller auftretenden Modifizierungen auf eine gemeinsame Ausgangsform zurückführen lassen, die sich noch heute bei einigen Arten in der Endodyogenie widerspiegelt.

Die reifen Merozoiten von *E. maxima* stimmen im wesentlichen in ihren Abmessungen und Ausdifferenzierungen mit den Verhältnissen bei anderen Eimeriiden überein. Bemerkenswert scheint dabei allerdings der Befund, daß das Conoid soweit vorgeschoben werden kann, so daß es schließlich dem Polring vorn aufsitzt, was seine Deutung als Penetrationsorganell im weiteren untermauert. Desgleichen konnten sehr enge strukturelle Verbindungen von Conoid, Rhoptrien (= Paariges Organell) und Mikronemen festgestellt werden, wodurch der von LEVINE (1970) vorgeschlagene Name »Apicomplexa« zur Kennzeichnung der »ehemaligen« Sporozoen als sehr zutreffend erscheint.

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Anschrift des Verfassers: Heinz MEHLHORN, D-5300 Bonn, Poppelsdorfer Schloß, BRD.

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# BIOLOGICAL RESIDUES IN MEAT AND MEAT PRODUCTS IN HUNGARY IN THE CALENDAR YEAR 1972*

By

J. TAKÁCS, Z. SIMONFFY and H. E. JANCSÓ

Central Laboratory (Head: J. TAKÁCS) of the Veterinary Meat Control Service, Budapest

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The Hungarian Veterinary Meat Control Service began its activity in detecting the chemical and biological residues in meat and meat products in 1971. The examinations of pesticide residues was decreased by the provision of 1/1970/I. 25. Ministry of Health and Ministry of Agriculture.

Besides meat and meat products our examinations for residues were carried out on group of pigs and beef-cattle origined from farming on a large scale.

To get enough information about the levels of residues in some animal population or in some consigment we used the composite sampling as recommended by the WHO.

Samples were taken from meat, fatty tissue, kidney and liver of swine and beef-cattle; from semi-preserved ham and shoulder; from winter and delicacy salami; from raw, cold smoked and dried sausages (Gyula) and edible lard.

Chlorinated hydrocarbon compounds and organic phosphorus insecticides were determined by thin-layer and gas chromatography, arsenicals by Gutzeit's-test, among heavy metals copper and zinc by the ditizon-titration method, and Zn-bacitracin by the agar-gel diffusion technique.

In 1972 725 different samples were analysed.

The results are demonstrated in Tables I—III.

*Examination of organic phosphorus insecticides:* Trichlorphon, Dichlorvos, Diazinon, Dimethoat, Malathion and Methylparathion.

In no case were organic phosphorus insecticides in 168 different meat and meat product samples detected.

## Conclusions

1. Groups of slaughter animals, derived from 59 state farms and farmers' co-operatives, were tested for the presence of biological residues.

*a)* There were significant differences in the amount of residues in meat samples obtained from different farms;

* Presented at the First International Congress For Animal Hygiene Budapest, Hungary 2—5 October 1973.

**Table I**

Average values of chlorinated hydrocarbon compounds

| Samples | | Basic amount of sampling | Total DDT (DDT+DDE+DDD) mg/kg | Total BHC ($\alpha + \beta + \gamma + \delta$), mg/kg |
|---|---|---|---|---|
| Pig | meat | 3 347 animals | 0.041 | 0.022 |
| | fatty tissue | 1 984 animals | 0.734 | 0.134 |
| | liver | 1 510 animals | 0.034 | 0.020 |
| Cattle | meat | 115 animals | 0.055 | 0.020 |
| | fatty tissue | 48 animals | 0.347 | 0.068 |
| | liver | 48 animals | 0.026 | 0.017 |
| Semi-preserved ham | | 185,957 pieces | 0.045 | 0.024 |
| Semi-preserved shoulder | | 64,717 pieces | 0.040 | 0.024 |
| Winter and delicacy salami | | 130,000 kg | 0.643 | 0.115 |
| Raw, cold smoked, dried sausage | | 190,000 kg | 0.547 | 0.094 |
| Edible lard | | — | 0.695 | 0.099 |

**Table II**

Average values for zinc, copper and arsenicals

| Samples | Zinc, mg/kg | Copper, mg/kg | Arsenicals, mg/kg |
|---|---|---|---|
| Porc | 24.2 | 0 | 0 |
| Pig liver | 52.5 | 11.2 | 0 |
| Cattle liver | 42.0 | 10.8 | 0 |
| Semi-preserved ham and shoulder | 20.2 | 0 | 0 |
| Winter and delicacy salami | 30.8 | 0 | 0 |
| Raw, cold smoked, dried sausage | 28.4 | 0 | 0 |

**Table III**

Zinc-bacitracin antibiotic

| Samples | Total number of samples | Negativ samples | Positive samples | |
|---|---|---|---|---|
| | | | < 0.5 | > 2 |
| | | | mg/kg | |
| Semi-preserved ham and shoulder | 47 | 37 | 10 | 0 |
| Pig liver | 2 | 2 | 0 | 0 |
| Pig kidney | 10 | 6 | 2 | 2 |

*b)* the pig fatty tissue contained more (0.734 mg/kg) total DDT than the cattle fatty tissue (0.347 mg/kg); perhaps due to the great difference in feeding between the two animal species;

*c)* the BHC values were approximately the same for swine and cattle, suggesting that BHC accumulated neither in vegetables nor in the animal body;

*d)* fatty tissue contained about ten-fold more DDT and about five-fold more lindan than meat;

*e)* according to our analyses chlorinated hydrocarbon compounds did not accumulate in liver tissue, therefore, the analysis of liver has to be carried out only in detection of acute poisoning.

2. The amount of chlorinated hydrocarbons in meat and meat products is much lower than the tolerated level accepted internationally.

3. Organic phosphorus insecticides were not detected at all in meat and meat products from large scale of samples, therefore, the practical significance of such analyses is low.

The presence of organic phosphorus insecticides in animal body and animal products was only expected in case of acute poisoning or if meat animals were treated with such preparations closely before slaughter.

4. The amount of zinc, copper, arsenicals as well as Zinc-bacitracin was not higher than the respective internationally accepted limit value.

5. The total amount of DDT decreased slowly in meat and meat products during the last years. During decomposition of DDT the amount of metabolites of DDE and DDD increased and therefore the total amount of DDT had been scarcely changed. The presence of DDT in animal tissues should be still taken into account for a long time.

## Summary

In the calendar year 1972 samples of meat and meat products were tested for the presence of biological active residues. Group of pigs and beef-cattle from large-scale farms were examined. Meat, fatty tissue, kidney and liver of meat animals and pasteurized ham and shoulder conserves, salami and raw, smoked, dried sausages were analysed.

Chlorinated hydrocarbon compounds, organic phosphorus insecticides, arsenicals, copper, zinc and Zn-bacitracin were examined in 725 samples. Results are presented in 3 Tables.

The DDT and Lindan content of fatty tissue was ten times and five times as high as the respective values for meat, however much lower than the accepted levels tolerated internationally.

Organic phosphorus insecticides were not detected at all in meat and meat products.

The level of heavy metals and trace elements as well as the Zn-bacitracin content was far below the accepted international limit value.

The total amount of DDT changed scarcely, therefore its presence should be taken into consideration for a long time.

The address of the authors: Dr. János TAKÁCS, Dr. Zoltán SIMONFFY, Edit JANCSÓ, 1095 Budapest, Soroksári út 58, Hungary.

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# INVESTIGATION OF THE CYTOTOXIC EFFECT OF F-2 TOXIN (ZEARALENONE) IN VARIOUS MONOLAYER CELL CULTURES

By

A. VÁNYI and Erzsébet SZAILER

Central Veterinary Institute (Director: A. VÁNYI), Budapest

(Received November 21, 1973)

Recently the F-2 toxin (zearalenone) produced by some *Fusarium* species in foodstuffs has caused serious animal-hygienic problems in Hungary. As it is well-known the "oestrogen syndrome" observed in young sows is due to the F-2 toxin (DANKÓ and ÁLDÁSY, 1969; DEBRECZENI and BORDA, 1972; DEBRECZENI and REJTŐ, 1969).

It has been proved that after a long-lasting feeding with F-2 toxin-containing foods the fertility of ganders and turkeycocks decreased or even stopped, depending on the quantity of the toxin (PALYUSIK et al., 1971). In these cases the quantity of the sperm and viability of spermatozoa were markedly reduced. There were many degenerated epithelial cells in the testicles, only a few spermatozoa were seen in the convoluted tubules, and even these were deformed. Turkeycocks proved to be less sensitive than ganders and the toxin did not influence the fertility of cocks (VÁNYI et al., 1973).

Foreign authors have reported in heifers and cows on reproductive-biological disorders which were attributable to the effect of F-2 toxin (ROINE et al., 1971). We have not found reliable data relating to bulls.

It is well-known that various mycotoxins have a cytotoxic effect (BODON and PALYUSIK, 1970; GEDEK, 1971; LEGATOR, 1966) which may be employed in the quantitative determination of these toxins.

As we had not reliable data about the cytotoxic effect of F-2 toxin, we investigated its influence on various cell cultures and the relationship between its biological and possible cytotoxic effects.

## Materials and Methods

The F-2 toxin applied in the experiments was obtained from toxin-producing *Fusarium graminearum* strains and purified by column and thin-layer chromatography. Being practically insoluble in water F-2 toxin (2000 $\mu$g) was dissolved in dimethylformamide (0.2 ml) and serial twofold dilutions were prepared of PBS (phosphate buffered physiological salt solution pH 7.2). To

eliminate the possible bacterial contamination, the solution was centrifuged at 7000 r.p.m. for 30 minutes. 2-2 tube cultures were inoculated with 0.2 ml quantity of each dilution. Controls were as follows: the dilution series of dimethylformamide made with PBS; PBS without dimethylformamide and non-treated cell cultures.

Cell cultures: primary cell monolayers were prepared from calf testicles, turkey testicles, cock testicles, swine testicles and chicken embryo fibroblasts; secondary monolayer cultures were prepared from swine thyroid glands.

Hanks' solution with 15% foetal calf serum and 0.5% lactalbumin hydrolysate was used as growth medium, and the same medium devoid of serum was used as maintenance fluid. The tube cultures were inoculated simultaneously with the medium-change and were incubated at 37°C. The results were read in the 6th, 16th, 24th and 48th hours after the inoculation.

## Results

The results about the cytotoxic effect of F-2 toxin are summarized in Table I.

It is clearly seen that all the swine, turkey and calf testicle monolayers treated with 500 or 250 μg/tube toxin had degenerated by the 16th hour, while only part of the monolayers treated with 125, 62.5 or 31.25 μg/tube showed signs of degeneration. The cock testicle, chick embryo fibroblast and swine thyroid gland monolayer cell cultures were not considerable affected even by the largerst dose. The cytotoxic effect did not change considerably between the 16th and 48th hours.

**Table I**

The cytotoxic effect of F-2 toxin in various monolayer cell cultures

| Dilution | Toxin, μg/tube | Degree of the cell-degeneration (in percentage) in the 6th and 16th hours after inoculation | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | swine-testicle | | turkey-testicle | | calf-testicle | | cock-testicle | | chicken embryo fibroblast | | swine thyroid gland | |
| | | 6 h | 16 h | 6 h | 16 h | 6 h | 16 h | 6 h | 16 h | 6 h | 16 h | 6h | 16 h |
| 1 : 1 | 500 | 100 | 100 | 100 | 100 | 100 | 100 | — | — | — | — | — | — |
| 1 : 2 | 250 | 100 | 100 | 100 | 100 | 50 | 100 | — | — | — | — | — | — |
| 1 : 4 | 125 | 50 | 100 | 50 | 80 | 25 | 50 | — | — | — | — | — | — |
| 1 : 8 | 63 | 20 | 50 | — | 50 | — | 25 | — | — | — | — | — | — |
| 1 : 16 | 31 | — | 10 | — | 5 | — | 5 | — | — | — | — | — | — |
| 1 : 32 | 16 | — | 5 | — | — | — | — | — | — | — | — | — | — |
| 1 : 64 | 8 | — | — | — | — | — | — | — | — | — | — | — | — |

In the control tubes containing only the dilution series of dimethylformamide made with PBS solution (Figs 1 and 2), the cells showed no lesions.

The swine testicle cell cultures appeared to be the most sensitive, followed by the turkey and calf testicle cells. The cock testicle cells proved to be strikingly insensitive.

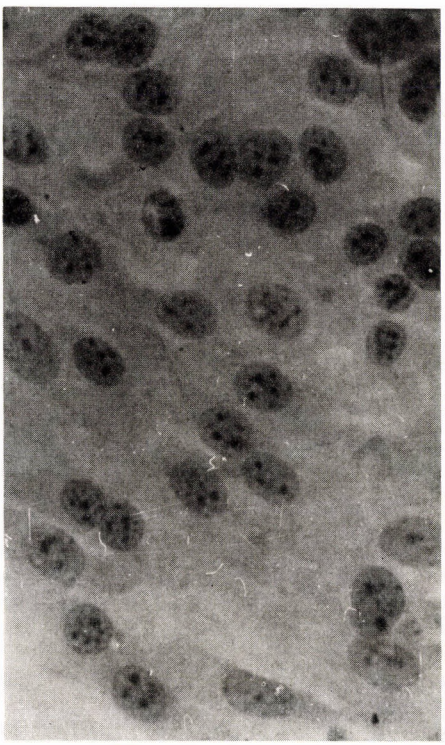


*Fig. 1.* Intact calf testicle monolayer cell culture in the 48th hour. (H. and E. stain ×600)

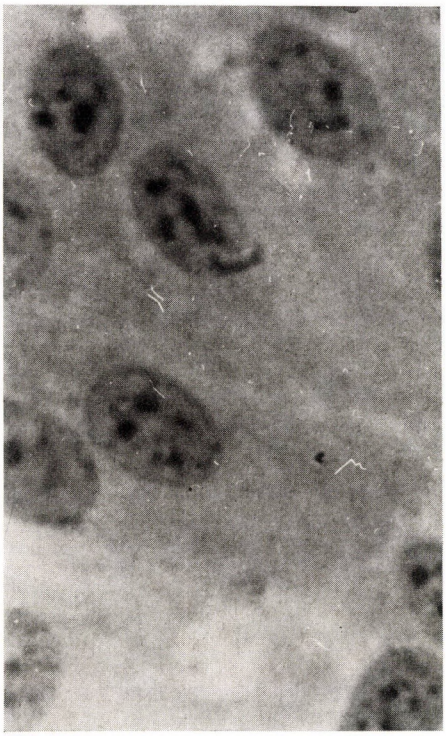


*Fig. 2.* Part of Fig. 1. (H. and E. stain ×1400)

## Discussion

The results of the present investigations have shown some correlation between the effect of F-2 toxin on cell cultures and its biological effect known in practice. The clinical signs and pathological lesions, caused by the F-2 toxin are localized to the genital organs, even there to certain kinds of tissue. The sensitivity of these tissues can be determined with histological examination even more evident. In sensitive animals the toxin damages the function of the uterus, ovary and testicle to a large extent. On this basis we supposed that the toxin-sensitivity of cell cultures is species-dependent. The study of the biological effect of F-2 toxin encounters many difficulties. Our former investigations

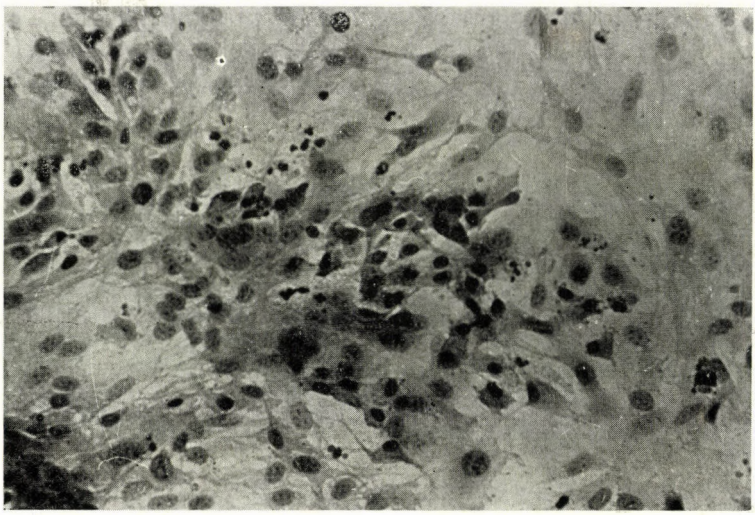


*Fig. 3.* Calf testicle monolayer cell culture in the 6th hour after treatment with F-2 toxin. (H. and E. stain ×240)

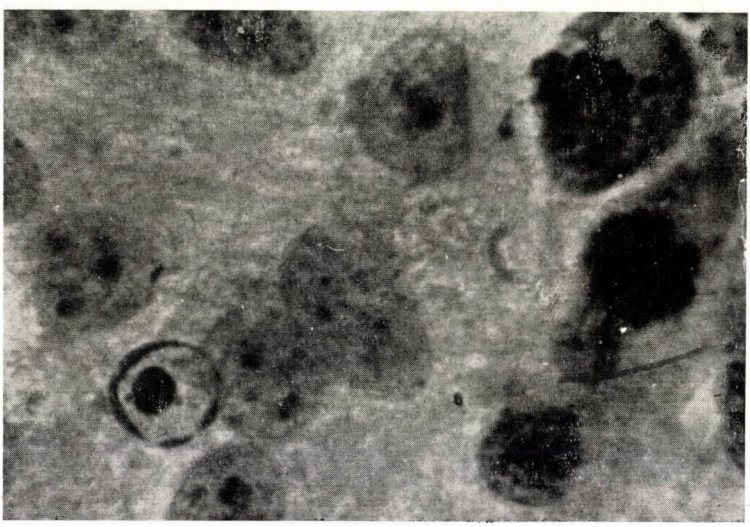
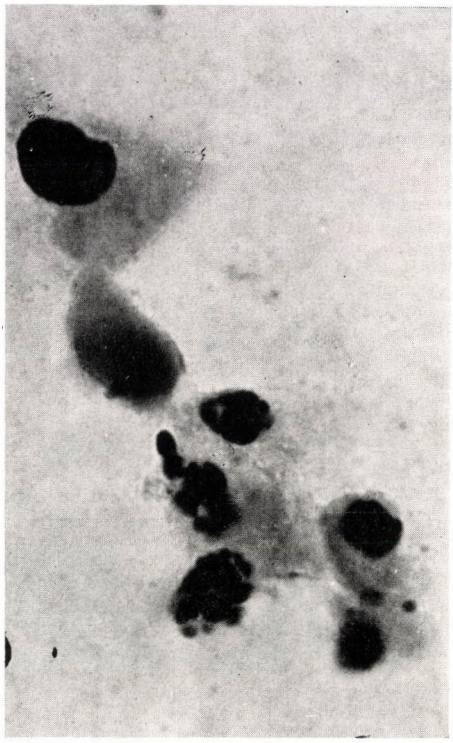


*Fig. 4.* Part of Fig. 3. The nucleus of cells degenerated under the effect of toxin is pycnotic or having karyorhexis, their cytoplasm stained darkly. (H. and E. stain ×1400)

concluded definitely to that the sensitivity of animal species varies on a large scale, therefore, the results of experiments on laboratory animals could not be referred to every species of animals. It was another difficulty that the examinations having been performed on large animals (swine, cattle) needed a great quantity of purified toxin, the production of which was troublesome. For the early investigations the testicle tissue of the toxin-sensitive species of





*Figs 5, 6.* Part of Fig. 4. (H. and E. stain ×1400)

animals was the most convenient. It appears from the experiments that there is a close correlation between the sensitivity of the monolayer testicle cell culture and that of the species from which the cell culture was prepared. It is in accordance with our observations that cock testicle cell cultures are not damaged by the F-2 toxin. On the basis of the obtained results, the cytotoxicity test is convenient for model experiment, by its help the sensitivity of the various species of animals and tissues against the F-2 toxin can be studied simply and rapidly. With this method we should like to obtain information relating to the mechanism of action of the toxin.

## Summary

The cytotoxic effect of F-2 toxin (zearalenone) was studied in various monolayer cell cultures. The testicle cell cultures prepared from animal species whose spermiogenesis is inhibited by the toxin proved to be sensitive to the toxin. The testicle cell cultures from a resistant species were not damaged by the toxin.

Other sorts of cell cultures showed no sensitivity to the toxin. The cytotoxicity test is convenient for the rapid determination of sensitivity to the F-2 toxin by various species of animals and tissues.

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The address of the authors: Dr. András VÁNYI, Dr. Erzsébet SZAILER, 1149 Budapest, Tábornok u. 2, Hungary.

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# COMPARATIVE STUDIES ON OPTIMAL CONDITIONS FOR ENTEROCOCCUS DEMONSTRATION*

By

J. TAKÁCS and S. KOVÁCS

Central Laboratory of Veterinary Meat Control Service (Head: J. TAKÁCS), Budapest

(Received January 2, 1974)

The streptococci belonging to Lancefield's D group, *viz.*, *Streptococcus faecalis* and its variants *Str. faecium*, *Str. durans*, *Str. bovis* and *Str. equinus*, are generally referred to by the collective term "faecal streptococci", because normally they occur in the digestive tract of man and warm-blooded animals. In a wider sense, however, the *Str. viridans* group and the atypical *Str. salivarius* and *Str. mitis* strains also come under the category of faecal streptococci. The latter two species occur chiefly in the mouth, but from there they gain access to the digestive tract and may constitute a dominant component of the gut flora. The systematic position of the atypical streptococci is intermediate between the viridans and enterococcus groups. Accordingly, these microorganisms are utilized in food microbiology as indicators of faecal contamination in food products (except for stale, non-stored or non-processed food). They may, along with other microbial indicators, detect faecal contamination in food production plants, objects and machinery and in the food products themselves, indicating the inadequacy of cleaning, disinfection and hygienic measures.

Examination of Lancefield's group-D streptococci from the point of view of food microbiology has shown that the resistance and growth properties of the species are variable. *Str. bovis* and *Str. equinus* are less resistant to heat treatment, freezing, drying and disinfection than the true enterococcus group, *viz. Str. faecalis* and its variants *(Str. zymogenes, Str. liquefaciens)*, *Str. faecium*, and *Str. durans*.

Faecal streptococci may also be utilized as indicators of the general microbiological quality and recently they have been reported to play a causal role in food poisonings. Furthermore, many enterococci may be present in foods without damaging the health of the consumer. The aetiological significance of enterococci in food poisonings has been a matter of dispute, because no experimental disease could be produced with them in man. Nevertheless,

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there is evidence that *Str. faecalis* and its variants may be held responsible for food poisonings under given conditions, the poisonous factor being either the living bacterium cell itself, or its toxic metabolic products, especially tyramine. Synergism with other microorganisms may be another cause of food poisonings by enterococci. At all events, foods having enterococcus counts above $10^6$/ml are hazardous to human health.

Although on the basis of resistance properties, growth ability and biochemical behaviour, enterococci form a fairly homogeneous group of streptococci, there are well defineable quantitative differences between species. It is known that *Str. faecalis* and its variants show the highest resistance, whereas *Str. faecium* is considerably less resistant. Nevertheless, enterococci generally correspond to the criteria established by SHERMAN (1937), *viz.*, they are thermoresistant (surviving a 30-minute heat treatment at 60 °C), can grow at 45 °C and pH 9.6, and in media containing 6.5% NaCl or 40% bile. SHERMAN's criteria are best met by *Str. faecalis* and its variants, *viz.*, several strains of *S. faecium* and *Str. durans* fail to grow at pH 9.6 or in media containing 6.5% NaCl.

Demonstration of enterococci is especially important in those cases in which heat treatment or pasteurization are sufficient to kill *E. coli* I, coliforms and *Enterobacteriaceae*, but fail to kill the more thermoresistant strains of enterococci which thus may become the single component of the surviving microflora along with the spores. Cooling and storage methods sufficient to kill or greatly reduce the more sensitive *E. coli* and coliform organisms, but not affecting enterococci, which are more resistant to cold, can reveal that faecal contamination had taken place before freezing. In such cases enterococci can serve as indicators of the possible presence of other similarly resistant pathogenic agents, as *e.g.* infections hepatitis virus, enteroviruses, etc. If enterococci are not found, it can usually be accepted that pathogenic or facultatively pathogenic *Enterobacteriaceae* are absent as well. Thus, the detection of enterococci is of particular importance in the case of baby foods.

As the species belonging to the enterococcus group are fairly heterogeneous in respect of certain properties, their reliable demonstration and evaluation is hampered by various difficulties. It therefore seemed important to establish the optimal conditions of isolation and propagation by comparative examinations of *Str. faecalis* and its two variants (v. zymogenes and v. liquefaciens), and *Str. faecium* and *Str. durans*. It is known that streptococci other than the above can also grow out if less selective media are used, whereas in highly selective media the growth of the less resistant enterococci, above all of *Str. faecium*, is suppressed. The demonstration of those enterococci which survive heat treatment and other technological processes depends on the degree of shock, the bacterium cells are injured by the shock and thus cannot grow in

the presence of bacteriostatic components in the medium. In those cases in which no Gram-negative microorganisms except enterococci are expected to survive, the isolation medium of choice is blood agar free from growth inhibitor.

## Experimental

*Material.* Liquid and solid media were used in the comparative studies as shown below.

A) *Liquid media*

Broth, containing sodium azide and glucose, formula I (MALLMANN and SELIGMANN, 1950)
Formula I + 0.003% bromothymol blue (RAJ, 1969)
Broth, containing sodium azide and glucose, formula II (HANNAY and NORTON, 1947)
Litsky—Mallman's broth (LITSKY et al., 1955)
SAE broth (PUSZTAI, 1969)
Broth, containing maltose and sodium azide (KENNER et al., 1961)
A common broth of uniform composition (basal medium, TAKÁCS, 1960) was used as reference medium throughout.

B) *Solid media*

Packer's agar (PACKER, 1943)
Maltose-azide agar (KENNER, et al., 1961)
Szita's agar (SZITA, 1957)
ZATK-agar (REUTER, 1968)
Barnes' agar (BARNES, 1956)
Slanetz—Bartley's agar (SLANETZ and BARTLEY, 1957)
SE-agar (RAJ, 1969)
Thallium-acetate-TTC-glucose agar (BARNES, 1956)
Kanamycin agar (HARTLEB, 1969)
TTC-glucose agar (THATCHER and CLARK, 1968)
A common agar of uniform composition (basal medium, TAKÁCS, 1960) was used as reference medium.

C) *Enterococci strains* used in the comparative studies:

O1 OKI 80 171 *Str. faecalis v. faecalis*
K1 Kiel 21 009 *Str. faecalis v. faecalis*
O2 OKI 80 172 *Str. faecalis v. liquefaciens*
J4 Sarajevo 3A *Str. faecalis v. liquefaciens*

K2 Kiel 293 *Str. faecalis v. zymogenes*
L4 NCTC 8176 *Str. faecalis v. zymogenes*
O4 OKI 80 174 *Str. durans*
L6 NCTC 8307 *Str. durans*
L5 NCTC 7379 *Str. faecium*
J5 Sarajevo T 70 *C Str. faecium*

*Strains used as controls:*

ATTC 6538 *Staph. aureus*
ATTC 9637 *E. coli*
ATTC 6633 *B. subtilis*

## Method

Before use the enterococcus strains were carried through several passages in the basal medium. One loopful (loop, 3 mm in diameter) of the last passage was inoculated into basal medium and incubated for 24—48 hours. Decimal dilutions were prepared with one ml of this culture (1 ml broth culture + 9 ml peptone-containing saline) up to $10^{-12}$. One ml of each of the dilutions ranging from $10^{-5}$ to $10^{-12}$ was inoculated into the liquid media listed above (10 ml medium was used for inoculation throughout), and the cultures were incubated for 24—48 hours at 37 °C. Final readings were made after 48 hours.

The inoculum for solid media was produced as above, and the same dilution range was used. Streak plates were prepared with 0.1 ml from each dilution. The results were read after incubation of the plates for 48 hours at 37 °C.

## Results

Culturing results in 16 different liquid and solid media and in the liquid and solid basal media are summarized in Table I, which also includes the data obtained in the optimal culturing media.

Table I in which the results are presented comparatively, clearly shows that among the liquid media tested, the 0.003% bromothymol blue-containing sodium azide-glucose broth proved to be most suitable for enterococcus demonstration. All enterococcus species grew well and reached the highest titres in this medium.

Optimal inhibitory effect was found in the SAE-broth, in which neither *Staph. aureus* nor *E. coli* was able to grow. *Staph. aureus* also failed to grow in the Litsky—Mallman broth, whereas the growth of *E. coli* was scarcely suppressed. The 0.003% bromothymol blue containing sodium azide-glucose

**Table I**

Culturing results in different liquid and solid media and in the liquid and solid basal media

| Media \ Strains | O1 OKI 80 171 *Str. faecalis var. faecalis* | K1 Kiel 21 009 *Str. faecalis var. faecalis* | O2 OKI 80 172 *Str. faecalis var. liquefaciens* | J4 Sarajevo 3A *Str. faecalis var. liquefaciens* | K2 Kiel 293 *Str. faecalis var. zymogenes* | L4 NCTC 8176 *Str. faecalis var. zymogenes* | O4 OKI 80 174 *Str. durans* | L6 NCTC 8307 *Str. durans* | L5 NCTC 7379 *Str. faecium* | J5 Sarajevo T70C *Str. faecium* |
|---|---|---|---|---|---|---|---|---|---|---|
| Basal medium (liquid) | 12** | 10 | 11 | 11 | 10 | 9 | 8 | 8 | 8 | 9 |
| Na-azide broth I | 12 | 12 | 11 | 11 | 10 | 9 | 10 | 9 | 9 | 9 |
| Na-azide broth II | 12 | 12 | 11 | 11 | 10 | 10 | 10 | 9 | 9 | — |
| Na-azide broth III | 10 | 10 | 9 | 12 | 11 | 12 | 10 | 10 | 8 | 8 |
| Litsky—Mallmann's broth | 12 | 12 | 11 | 11 | 10 | 10 | 9 | 8 | 9 | 9 |
| SAE broth | 12 | 9 | 11 | 11 | 9 | 10 | 9 | 10 | 9 | 7 |
| Maltose-azide broth | 12 | 12 | 11 | 11 | 9 | 9 | 10 | 10 | 9 | 9 |
| Basal medium (solid) | 9 | 9 | 8 | 9 | 9 | 11 | 8 | 9 | 9 | 9 |
| Packer's agar | 8 | 9 | 8 | 9 | 9 | 10 | 8 | 8 | 9 | 8 |
| Maltose-azide agar | 9 | 9 | 8 | 9 | 11 | 11 | — | — | 9 | — |
| Szita's $E_{67}$ agar | 9 | 9 | 9 | 9 | 9 | 11 | 7* | 9* | 9* | 8* |
| ZATK agar | 10 | 9 | 9 | 9 | 9 | 11 | — | — | 9 | — |
| Barnes' agar | 9 | 9 | 8 | 9 | 9 | 11 | — | 8 | 9 | — |
| Slanetz—Bartley's agar | 10 | 9 | 9 | 9 | 9 | 11 | — | — | 9 | — |
| SE-agar | 8 | 8 | 8 | 9 | 9 | 8 | 8 | 8 | 9 | 9 |
| Thallium acetate-TTC-glucose agar | 9 | 8 | 8 | 9 | 9 | 11 | — | — | 9 | — |
| Kanamycin agar | 8 | 8 | 8 | 8 | 8 | 8 | — | 8 | 9 | 9 |
| TTC-glucose agar | 9 | 9 | 8 | 9 | 9 | 11 | — | — | 9 | — |

Explanation: ** Germ counts expressed as base-10 logarithmic values
* colony formation not characteristic
— no outgrowth

broth had no outstanding inhibitory properties, but could be very easily prepared which is important in routine work and furnished favourable conditions for an even, ready growth of all enterococcus strains.

Among the solid media Packer's agar proved to be the medium of choice for enterococcus demonstration; this was the only medium on which all examined enterococcus strains were able to grow.

Both *Str. faecium* strains, but especially the one designated J5 Sarajevo T 70, either failed to grow or did not grow characteristically on maltose-azide

**Table II**

Tests recommended for the identification of enterococci

| | *Str. faecalis var. faecalis* | *Str. faecalis var. zymogenes* | *Str. faecalis var. liquefaciens* | *Str. faecium* | *Str. durans* | *Str. bovis* | *Str. equinus* |
|---|---|---|---|---|---|---|---|
| Growth at 45 °C | + | + | + | + | + | + | + |
| Growth at 50 °C | − | − | − | + | +/− | − | − |
| Growth at pH 9.6 | + | + | + | + | + | − | − |
| Growth in presence of 6.5% NaCl | + | + | + | + | + | − | − |
| Growth in presence of 40% bile | + | + | + | + | + | + | + |
| Survival at 60 °C for 30 min | + | + | + | + | + | − | − |
| Reduction of TTC | + | + | + | − | +/− | +/− | − |
| Liquefaction of gelatine | − | −/+ | + | − | − | − | − |
| Growth in medium containing 0.05% K-tellurite | + | + | + | − | − | − | − |
| Decomposition of sorbitol | + | + | + | +/− | − | − | − |
| Decomposition of arabinose | − | − | − | + | − | −/+ | − |

Symbols used: + positive; − negative; +/− changing, chiefly positive; −/+ changing, chiefly negative

agar, Szita's agar, ZATK agar, Barnes' agar, Slanetz—Bartley's medium thallium acetate-TTC-glucose agar and TTC-glucose agar, developing only on kanamycin agar, SE agar and Packer's agar.

The *Str. durans* strains did not grow on maltose-azide agar, ZATK agar, Slanetz—Bartley's agar, thallium acetate-TTC-glucose medium and TTC-glucose medium.

The requirement of food microbiology, *viz.*, that the medium should support the growth of all enterococcus strains was met only by Packer's agar and the SE agar. Since, however, the SE agar does not contain any inhibitor, its use should be completed by the oxacillin test.

## Discussion

On the basis of comparative studies, sodium azide-glucose broth containing 0.003% bromothymol blue is recommended as the optimal enrichment group. Streak plates from this culture should be prepared on Packer's agar medium to confirm the diagnosis. The incubation time is 24—48 hours at 35—37 °C. This procedure should be employed in investigations aimed at exclusion of the presence of enterococci.

The criteria of classification into the enterococcus group are among others growth ability in the above two media, the formation of chains consisting of 4–5 streptococci, elongate in the axis of the chain, on Packer's agar and negative catalase reaction of the colonies.

The above criteria and heat resistance test for 30 minutes at 60 °C have usually been sufficient for routine identification of enterococci. However, classification and proper identification require the performance of all tests listed in Table II.

## Summary

In food microbiology, enterococcus demonstration has chiefly served as an indicator of faecal contamination. However, enterococcus strains, above all *Str. faecalis*, have several times been found to be responsible for food spoilage and causation of enteric disease.

The various species of enterococcus differ considerably in respect of resistance, biochemical properties and growth ability on selective and other media. Comparative studies were performed on 6 liquid and 10 solid media recommended for the cultivation of enterococci, to find the most suitable medium for isolation.

Among the liquid media tested, the sodium azide-glucose enrichment medium containing 0.003% bromothymol blue proved to be the medium of choice, whereas among the solid media Packer's agar was found to be the best. Optimal culturing results were obtained in both media on incubation for 24—48 hours at 35—37 °C.

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Address of the authors: Dr. János TAKÁCS, Dr. Sándor KOVÁCS, 1095 Budapest, Soroksári út 58, Hungary

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# SEROLOGICAL EXAMINATION OF *E. COLI* STRAINS ISOLATED FROM CALVES

By

J. VARGA and A. F. FARID*

Department of Epizootology (Head: J. MÉSZÁROS),
University of Veterinary Science, Budapest

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Enteric diseases are known to cause great losses in large-scale cattle stocks. In Hungary, the country average of losses among less than two week old calves varied between 5.2 to 6.8%, and 57.1 to 72.4% of the deaths were due to enteric diseases, above all to *E. coli* diarrhoea (MÉSZÁROS, 1972).

Apart from predisposing factors, various serotypes of *E. coli* have a great aetiological significance in *E. coli* diarrhoea. Although the number and distribution of the pathogenic serotypes varies by countries, generally only a few types are involved in the aetiology. ÁLDÁSY (1955, 1959) isolated chiefly strains belonging to serogroups O9, O41, O78 and O101 from the carcases of calves died of *E. coli* diarrhoea. Other authors encountered partly the former O groups, partly others, *viz.* WRAMBY (1948) found O15, O8: K20 and O9: K30; ULBRICH (1954) O9, O8, and O78, the antigens K7, K14 and K30 within the serogroup O9 and either K11 or K56 within serogroup O8; FEY (1957a, b) groups O78: K80, O115 and O86; DAM (1960) the groups O78; GUINÉE (1963) groups O15, O8 and O9; GOSSLING et al. (1964) groups O9, O101 and O15; SOJKA (1965) groups O8, O15 and O26; SÖDERLIND (1965) groups O78, O15 and O115; WILLINGER and MATHOIS (1965) groups O78, O9 and O15; RADOMINSKI and KONRACKI (1967) groups O9, O101 and O78; GLANTZ et al. (1962) groups O117 and O101; MANZ (1971) groups O78, O8 and O20. Strains isolated in Hungary from diseased calves have not been examined for K antigens.

It seemed therefore worth-while to examine the distribution and frequency of occurrence of O and K antigens in those *E. coli* strains which have recently been responsible for disease of calves in Hungary.

## Materials and Methods

A total of 288 strains were isolated from carcases of 117 suckling calves, originating from 91 large-scale farms. All calves died either at, or under, two weeks of age. Isolation was usually attempted from the mucosa of the small intestine and from mesenteric lymph nodes; from organs only in those cases in which there was suspicion of septicaemia. The greater part of the strains were isolated in this laboratory, the others were received through the courtesy

* On leave from the Veterinary Laboratory and Research Institute, Ministry of Agriculture, DOKKI, Cairo, U.A.R.

of the Veterinary Institute, Miskolc, and the Central Veterinary Institute, Budapest.

The isolates were identified on the basis of biochemical properties, and the presence of O and/or K antigens, using KAUFFMANN's (1966) method. Biochemically, the strains were tested for nitrate reduction, lactose fermentation, urease, indol and hydrogen sulphide production, positive Voges—Proskauer and methyl red reaction, citrate utilization, and liquefaction of gelatine. The O antigens were demonstrated by means of 57 O and OK sera, *viz.* O2-O5, O7-O10, O11, O13, O15, O17, O20, O21, O23, O25, O28, O41, O44, O56, O69, O75, O78, O86, O93, O101, O111—O113, O115—O117, O119, O124—O126, O128, O136—O139, O141—O157.

As to K antigen determination, all known K sera (1—94) were at our disposal except K65, K90 and K92. Both O and K sera were produced with standard *E. coli* type strains, supplied from the National Institute of Public Health, Budapest, and from the International Escherichia Centre, Copenhagen.

O groups were determined with heated antigens, K antigens with live cultures, first by slide test, then by titration in tubes. In the slide agglutination both O and K sera were used in a pooled form. The K pools were prepared by the method of EWING et al. (1956), slightly modified by us. For production of O and K antigens, the strains were cultivated on the $D_{1,5}$ medium of SCHLECHT and WESTPHAL (1966), containing 0.1% glucose.

The O and K antigens of the isolates were regarded as identified if the strain under study was agglutinated up to the titration end points of the corresponding O and K sera.

## Results

The biochemical properties and the grouping of the isolates are shown in Table I. All isolates except two could be classified into the genera *Escheri-*

**Table I**
Biochemical properties* of the isolates

| Genus | No. of strains | Nitrate | Lactose | Indol | Urease | Gelatin | $H_2S$ | $NH_4$-citrate | Voges-Proskauer | Methyl red |
|---|---|---|---|---|---|---|---|---|---|---|
| *Escherichia* | 263 | + | + | + | − | − | − | − | − | + |
| | | | | | (4+) | (4+) | (16+) | (5+) | | |
| *Klebsiella* | 15 | + | + | − | + | − | − | + | + | − |
| *Proteus* | 8 | + | − | + | + | + | + | + | − | + |
| | | | | (3−) | | | | (2−) | | |

* The figures in brackets indicate the number of strains behaving differently as characteristic of the type

*chia*, *Klebsiella* or *Proteus*. The overwhelming majority of the 263 *E. coli* strains showed the biochemical reactions characteristic of the genus, but a few strains reacted differently in certain tests, producing urease, or $H_2S$, liquefying gelatine, or growing in citrate medium. The *Klebsiella* strains showed the characteristic biochemical reactions without exception, their colonies were all markedly mucous and their external appearance was in every respect similar to the colonies of *E. coli* bacteria containing K(A) antigen. Eight isolates were found to belong to the *Proteus* genus, five being identified as *Proteus* vulgaris and three as *Proteus* mirabilis.

Data on the antigenic patterns of the *E. coli* strains are shown in Table II. Of the strains identified biochemically as *E. coli*, 150 (57%) were typeable with the 57 O and OK sera listed above. The groups O101, O20, O41, O8 and O7 were encountered most frequently and except for two strains the *E. coli* isolates belonged to 11 O groups. All colonies of strains O101 and O41 and part of the colonies of strains O8 and O9 had a markedly viscuous and mucoid appearance. Investigations into the K antigens have revealed that these strains

**Table II**

O and K antigens of *Escherichia coli* strains isolated from suckling calves

| O antigen | No. of strains | K antigen | No. of strains | No. of strains with unidentifiable K antigen |
|---|---|---|---|---|
| O101 | 50 | K28(A) | 17 | 17 K(A) |
| | | K30(A) | 16 | |
| O20 | 19 | . | | 19 |
| O41 | 13 | . | | 13 K(A) |
| O8 | 12 | K28(A) | 5 | |
| | | K71(B) | 5 | 2 |
| O7 | 12 | . | | 12 |
| O21 | 9 | . | | 9 |
| O23 | 9 | . | | 9 |
| O153 | 8 | K71(B) | 2 | 6 |
| O9 | 7 | . | | 3 K(A) |
| | | | | 4 |
| O78 | 6 | K80(B) | 6 | |
| O75 | 3 | K— | 1 | |
| | | . | | 2 |
| O17 | 1 | . | | 1 |
| O117 | 1 | K15(L) | 1 | |
| Totals | 150 (57%) | | 52 (34.6%) | 98 (65.4%) |

. Nature of K antigen undetermined
K—, K antigen lacking

contain K(A) antigens. The majority of the strains belonging to serogroup O101 contained one of two K(A) antigens, either K28 or K30, at roughly identical proportions, whereas the remaining strains, although these also bore a thermostable surface antigen, were not typeable with any of the known K(A) sera. Apart from the strains O101, K(A) antigen also occurred in strains O8, O41 and O9 but only the K(A) antigen of some O8 strains could be identified, as O8 : K28(A). The other part of the strains classified on the basis of O antigens bore either K(B) or K(L) antigen, *viz.* part of the strains O8 and O153 the antigen K71(B) and all strains O78 bore K80(B). K(L) antigen could only be identified in a single strain, in the combination O117 : K15(L). Although except for a single strain O75 : K- all *E. coli* isolates identified by their O antigen contained some kind of K antigen (being inagglutinable with the homologous O sera in the living state), the latter could be determined only in 34.6% of the strains.

Like in the O antigen determinations, several strains were found to cross-react with various K sera but the cross-reaction titres never reached the end titre of the serum.

## Discussion

The majority of those *E. coli* strains which cause disease in suckling calves form intransparent mucoid colonies on solid medium. The biochemical properties of a few strains differ from those generally characteristic of the genus.

Strains belonging to serogroups O101, O20, O41, O8 and O7 are responsible for disease in calves most frequently but strains of other serogroups, such as O21, O23, O153, O9 and O78 could also be found. The isolates were found to bear K antigen, almost half of them K(A), occurring chiefly in the patterns O101 : K28(A), O101 : K30(A) or O8 : K28(A). Some strains of other O serogroups bearing thermostable antigen, probably K(A), were also found. The other part of the strains bore either K(B) or K(L) antigen most of these antigens, however, could not be identified.

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## Summary

Two hundred and eighty-eight coliform strains, isolated from carcases of 117 calves died of *E. coli* diarrhoea in 91 large cattle farms, were examined for biochemical behaviour and O and K antigens. Fifty-seven *E. coli* O and OK sera were used for determination of O antigens and all (1—94), except three *E. coli* K sera for determination of K antigens.

On the basis of biochemical examinations, 263 isolates were identified as *E. coli*, 15 as *Klebsiella*, 5 as *Proteus vulgaris*, 3 as *P. mirabilis*. The remaining two strains could not be classified into the above genera. The greater part of the *E. coli* strains showed the biochemical behaviour generally characteristic of the genus, but a few strains behaved differently in certain reactions.

A total of 150 strains (57%) could be classified into O groups with the available range of sera. The greater part of the strains belonged to the serogroups O101, O20, O41, O8 and O7, whereas the groups O21, O23, O153 O9 and O78 were relatively rare.

All serologically identified strains, except one, were found to bear K antigen. Almost half of the strains bore the antigen K(A), occurring in patterns O101 : K28(A), O101 : K30(A) and O8 : K28(A), but not nearer identifiable thermostable, probably K(A) antigens were also found in strains belonging to serogroups O9 and O41. The other part of the strains possessed either K(B) or K(L) antigens, as a rule in the patterns O8 : K71(B), O153 : K71(B), O78 : K80(B) and O117 : K15(L).

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Address of the senior author: Dr. János VARGA, 1581 Budapest, P.O.B. 22, Hungary

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# THE IMPORTANCE AND HISTOPATHOLOGY OF HYPOTHALAMIC CHANGES IN ANIMALS INOCULATED INTRACEREBRALLY WITH HŐGYES' FIXED STRAIN OF RABIES VIRUS

By

D. KARASSZON

National Institute of Public Health (General Director: B. TÓTH), Budapest

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Detailed information about the normal and pathologically altered functions of the hypothalamus has chiefly been obtained from stereotactic stimulation experiments (HESS, 1954, 1956). Investigations with electric stimulation, electrocoagulation, various chemical stimulants as well as ultrastructural studies and research on vegetative functions, endocrinic mechanisms and behavioural reactions have completed the general knowledge of neoplastic, traumatic, toxic and inflammatory diseases of the hypothalamus (KLAGES, 1960).

In the course of studies on the pathogenesis of experimental infection of animals with Hőgyes' fixed strain of rabies virus, it was soon realized that the hypothalamus becomes more affected than other parts of the central nervous system. Further study of this phenomenon was supposed contribute to the pathogenesis of rabies as well as to the pathophysiology of the hypothalamus.

## Materials and Methods

*Virus.* Material from the 2194th rabbit brain passage of Hőgyes' fixed strain of rabies virus was kindly supplied by the Phylaxia Biologicals and Feedstuffs Co., Budapest. The freeze-dried rabbit brain was made up to obtain a 10% suspension in distilled water and was administered intracerebrally to experimental animals in doses of 0.02 (monkeys, rabbits) or 0.01 ml (mice, hamsters). The sheep brains were supplied from the vaccine production lots of Phylaxia. The virus content of the basic material was $10^6$ intracerebral mouse $LD_{50}/g$.

*Experimental animals.* The rhesus monkey, sheep and albino mouse brains originated from earlier studies (KARASSZON, 1969, 1970, 1971). In addition, five Syrian gold hamsters and five rabbits were infected intracerebrally, using the technique previously reported. All experimental animals originated from the stock of the National Institute of Public Health.

*Histological preparations.* Brain and spinal cord of animals spontaneously died or killed in a certain phase of the illness were fixed in 10% formalin solution and embedded in paraffin. Frontal (transversal) 10—30 $\mu$ thick sec-

tions were cut and stained with haematoxylin and eosin, chromalum-gallocyanin, van Gieson's technique and Luxol fast blue. Care was taken that the sections include areas of the anterior, middle and posterior parts of the hypothalamus.

## Results

*Monkey*. The rhesus monkeys died with characteristic symptoms of rabies on intracerebral infection showed acute inflammatory changes in the hypothalamus (Fig. 1).

On the basal surface of the brain the areas surrounding the hypothalamus were widened owing to serous-cellular infiltration, and the vessels in these areas had markedly distended lumina.

Sections from the anterior part of the hypothalamus showed marked vascular changes. Especially the small vessels and capillaries were distended, with swollen endothelial cells and activated adventitial cells. Activation was manifested by a marked mitotic activity; perivascular infiltration did not take place.

The texture of the brain tissue became loose in the perivascular areas and around the third ventricle.

A diffuse microglial proliferation was found nearby the vessels. Glia cell foci were rarely encountered.

Nerve cell lesions were chiefly found in the supraoptic and paraventricular nuclei. Less pronounced degenerative changes, such as hyperchromasia, shrunken nuclei and cytoplasmic vacuolization occurred in almost all groups of hypothalamic nuclei. In places glia cells made appearance around the degenerating nerve cells, but neuronophagia could not be observed.

Sections cut in level with the tuber showed activation of endothelial and adventitial cells, perivascular edema, mild, diffuse microglial proliferation and degenerating nerve cells. Some of the latter disappeared completely, their places being marked by roundish gaps, arisen from distension of the intercellular space. Changed neurons were found in the ventromedial, lateral and infundibular nuclei alike, but there was no indication of neuronophagia.

The vascular changes, diffuse glia cell proliferation and neuronal lesions found in the caudal area appeared to be more severe than the similar changes in the supraoptic region. Certain fields showed a variegated appearance at low magnification, owing to the variously altered staining properties of nerve cells. The cytoplasmic vacuolization of nerve cells was particularly conspicuous in these preparations.

*Sheep*. Brains from sheep inoculated intracranially (subdurally) and exsanguinated in the terminal stage of the disease showed marked inflammatory lesions in the hypothalamus. This area presented the picture of an active

vascular hyperaemia of the fluxion type in contrast to the generally congestive appearance of vessels in other parts of the brain.

The vascular endothelial cells were swollen and the adventitial cells were in an excited state. Consequently, ringlike perivascular infiltration, consisting of 1 or 2 rows of round cells, developed around some small vessels. Most round cells were of the lymphocyte type; histiocytes and monocytes were quite rare. Loose small foci, consisting of microglia cells, appeared along the vessels and merged to form microglia cell infiltration in larger areas.

In sections cut at different planes, the nerve cells of the brain nuclei showed degenerative changes of varying number and type. The main changes were shrinking of the nuclei, which thus assumed bizarre shapes, and marked vacuolization of the cytoplasm. Glia cells were also found in varying numbers in certain brain nuclei, but their presence was sometimes unrelated to the degenerative neuronal changes. Neuronophagia could not be observed.

The changes of the different hypothalamic nuclei did not notably differ in severity. Degenerative neuronal changes and reactive gliosis were uniformly found in the supraoptic, paraventricular, ventromedial etc. nuclei. The only difference was that glia cell reactions predominated in the anterior part, whereas vascular reactions in the posterior part.

*Gold hamster.* The hypothalamus of the Syrian gold hamsters which died of rabies after intracerebral inoculation showed serous-cellular infiltration of the soft meninges, especially in the basal parts of the brain. In this areas the meningeal vessels were markedly dilated, the lumina were packed with red blood cells, the adjacent parts of the meninges were edematous and infiltrated by a few round cells.

In comparison to changes in other brain areas, the great severity of the acute inflammatory lesions in the hypothalamus was quite conspicuous. Chiefly the posterior parts appeared to be involved, although the supraoptic and tuberal areas were also affected. Cellular changes (hyperchromasia of nuclear membrane, karyopyknosis, chromatolysis and marked cytoplasmic vacuolization) were found not only in the mamillary area, but also in the supraoptic, ventromedial, infundibular etc. regions. Apart from neuronal changes, glia cell and vascular lesions of the type described in the foregoing also occurred in hamster brains, accompanied by pericellular oedema and plasmorrhagia. As to the degree of injury in the different regions, lesions of the caudal part seemed to be the most severe.

*Mouse.* Acute inflammatory lesions of the hypothalamus were easily recognized and were more severe than the changes in other brain areas of mice died in consequence of the experimental infection. The relative severity of neuronal, mesenchymal and glia cell changes was variable in the different hypothalamic areas. Either the one, or another component of the inflammatory-degenerative complex was predominant.

In the nuclei of the oral part, the diffuse multiplication of glia cells was the main feature (Fig. 2), the vascular and degenerative neuronal changes being chiefly found in the posterior regions. The staining affinity of nerve cells varied even within one and the same hypothalamic nucleus thus imparting a variegated appearance to the picture.

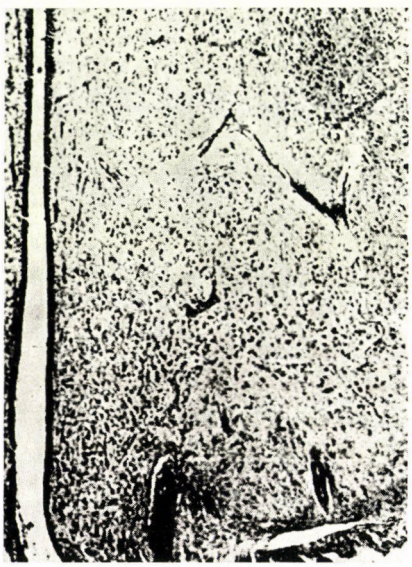

*Fig. 1.* Perivascular infiltrations and diffuse glia cell proliferation in the hypothalamus of a rhesus monkey died in experimental rabies after infection with fixed virus. Gallocyanine stain, × about 18

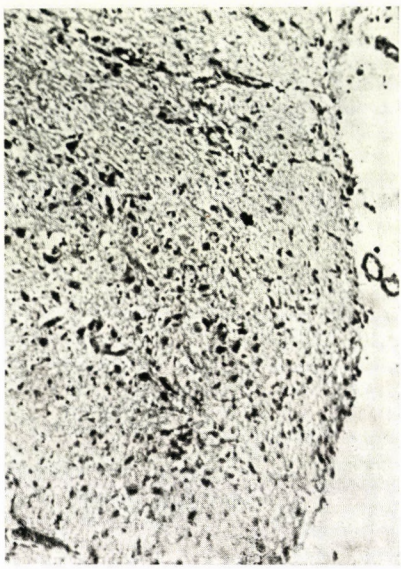

*Fig. 2.* Diffuse microglia proliferation in the hypothalamic supraoptic nucleus of a mouse died of rabies after experimental infection with fixed virus. Gallocyanine stain, × about 60

Some cells showed chromatolysis, shrinking of the nucleus, others were markedly swollen, showing a reticulating, net-like pattern of degeneration in the Nissl-region or, in more severe cases, a marked cytoplasmic vacuolization. In places glia cells made appearance between the affected nerve cells, whereas the glia cell reaction was absent elsewhere. No indications of neuronophagia were found. The vessels showed an active hyperaemia, especially in the caudal parts. The adventitial cells were in a state of activation, but perivascular cuffing was not observed.

*Rabbit.* The first rabbit killed on the fifth day after experimental infection *i.e.* on the first day of clinical illness, showed acute inflammatory lesions in the hypothalamus. The main changes were vascular dilatation, congestion of the lumina with red blood cells, swelling of endothelial cells and activation of adventitial cells (Fig. 3). Glia cells scarcely showed signs of activation,

if any. Neuronal changes were also mild and were practically limited to the paraventricular and supraoptic nuclei.

The second rabbit was killed on the seventh day after experimental infection, this was the second day of manifest disease. Acute inflammatory and degenerative lesions were equally found in the anterior and posterior parts of the hypothalamus.

Further two rabbits were sacrificed on the 8th day after infection, *i.e.* on the third day of disease. The fifth rabbit, which had been infected intracerebrally along with the rest of the group, still appeared healthy and was exsanguinated on the 9th day.

The two animals killed after a 3-day course of illness showed advanced inflammatory lesions in the hypothalamus. The vessels were dilated, having swollen endothelial cells, and thrombus formation was also found in several small vessels. The adventitial cells were in a state of activation, several mitotic and bizarre-shaped shrunken forms were found among them and in places karyorrhexis was seen.

The hypothalamic nuclei showed degenerative changes. The main changes were tigrolysis, central chromatolysis, alterations of nuclear structure, karyopyknosis, cytoplasmic vacuolization and pericellular oedema (Fig. 4).

Although the lesions appeared very severe at first sight, these regressive neuronal changes usually did not lead to a complete nerve cell destruction and loss of neurons. Even degenerative changes of the neurons were not always accompanied by an energetic glia cell reaction. The multiplication of microglia cells resulted as a rule in a loose, diffuse infiltration. Focus formation was exceptional.

In the hypothalamus of rabbits exterminated after several days of illness, diffuse glia cell proliferation predominated in the anterior part of the hypothalamus, whereas acute neuronal and vascular lesions were chiefly found in the posterior part.

The histopathological study of brain and spinal cord from the rabbit which did not show symptoms until killed on the 9th day after experimental infection requires further investigations.

## Discussion

Apart from the usual experimental approach to nerve function studies (stereotactic stimulation and destruction, fibre degeneration, treatment with ultrasound, chemical agents, X-ray, etc.), certain viruses may be utilized in experimental neurology. In our opinion, the neurotropic fixed rabies virus might especially serve the purpose, because recent research results (Baer, Shanta and Bourne, 1968; Petrovic and Timm, 1969; Schneider, 1969;

SCHNEIDER and SHOOP, 1972) have unequivocally confirmed that it spreads exclusively along the nerve paths.

If the mode of spread is the same also in the central nervous system (SCHNEIDER and BURTSCHER, 1967), the cell changes caused by the virus may be useful in revealing pathes, anastomoses and functional relations.

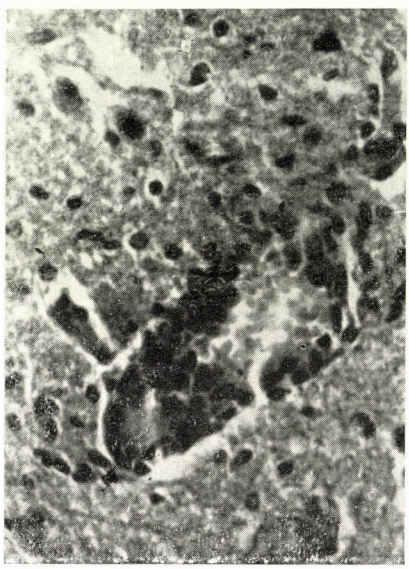

*Fig. 3.* Vascular lesions in the anterior part of rabbit hypothalamus. HE stain, × about 120

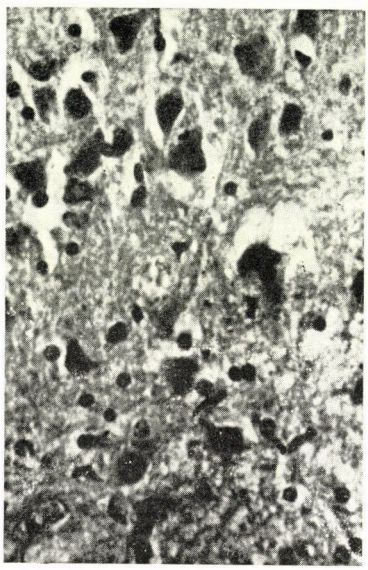

*Fig. 4.* Degenerative nerve cell changes in the dorsomedial nucleus of rabbit hypothalamus HE stain, × about 300

It was expected that the present study would, apart from the neurological information, disclose some new aspects of the pathogenesis of rabies as well. The effect of rabies virus on the hypothalamus was investigated first with the aim to throw more light on this long-disputed problem.

The importance of lesions in the diencephalic autonomous centres was pointed out by SLOTWER already in 1926, and was subsequently confirmed by KROLL (1927) and TARISKA (1941), but SHÜKRÜ AKSEL (1958) failed to find sufficient proof to the elective involvement of the autonomous nervous system in studies on 93 humans and 362 animals of various species (dog, cat, rabbit, jackal, calf, donkey).

In the meantime, extensive studies on hypothalamic structure and function resulted in the identification of the areas responsible for the symptoms of autonomous nerve function disorders (HESS, 1954, 1956; MAGOUN et al., 1938; GLEES, 1961; etc). This necessitated a revision of our knowledge on the pathomechanism of rabies.

The present findings show in accordance with our earlier observations that on intracerebral administration to experimental animals, Hőgyes' fixed strain of rabies virus causes a characteristic encephalitis. This accords well with the observations on other fixed virus strains BINDRICH, 1956; BINDRICH, POTEL and KUWERT, 1958; HURST and PAWAN, 1931; JOHNSON and LEACH, 1940; JOHNSON, 1965; JACOTOT and ROUX, 1937; NICOLAU et al., 1962; POTEL, 1958; THIERY, 1960).

The various species of experimental animals (rhesus monkey, sheep, Syrian gold hamster, albino mouse and rabbit) showed essentially the same type of encephalitic reaction, but the acute process, consisting of neuronal, glial and mesenchymal components, differed by the various regions in the central nervous system in respect of severity and nature of the main component.

Of course, the lesions found are in themselves not characteristic of rabies; similar changes can occur in other brain areas or in encephalitides caused by other viruses.

The severity of the changes could well be held responsible for the characteristic symptoms. The lesions found in the supraoptic and paraventricular nuclei can account for the fever which regularly accompanies infections, by fixed and street viruses (LŐTE, 1887; HŐGYES, 1897; AUJESZKY, 1921). Piloerection was observed by REMLINGER and BAILLY (1942) and BRANDENBURG (1952) as an early symptom of rabies in mice, and sympathicotonia was mentioned by JOHNSON (1965) in the pathogenesis of rabies. It should be noted that NICOLAU et al. (1962) also regarded the supraoptic nucleus as the main site of changes in rabies.

The microscopic lesions found by us in the lateral hypothalamic regions, in accordance with certain experimental neurological findings may have a certain bearing on the forced sniffing of the animals, often observed by us and others. Involvement of the lateral nucleus may also be held responsible for inappetence and rapid loss of condition, which are regular symptoms of fixed virus infections, as shown by JOHNSON and LEACH (1940) in mice, by AUJESZKY (1932) in rabbits and by ourselves (KARASSZON, 1969) in rhesus monkeys. The simultaneous dilatation of the pupils and of the palpebral fissure, retraction of the third eyelid, photophobia, extrasystolic tachycardia and an increased defense reaction indicate that after the lateral nucleus the tuber cinereum also becomes involved. This is further shown by hyperglycaemia and glycosuria (REMLINGER and BAILLY, 1942) and alteration of the blood picture *viz.* neutrophilia and lymphopenia and appearance of myelo- and metamyelocytes during the paralytic stage.

The inflammatory and degenerative changes of the ventromedial and infundibular nuclei were severe enough to acoount for general excitation, for the priapismus, especially in albino mice, the marked exophthalmus in monkeys, the increased tachycardia, quick respiration and for the perverse ap-

petite sometimes causing the animals to devour indigestible objects (RÁTZ, 1910).

The microscopic lesions found in the posterior hypothalamic areas can easily be linked with the paroxysmal tachypnoea, listlessness, fatigue, somnolence and finally stuporous state, which regularly developed with the progression of the experimental disease.

It should be mentioned that the symptoms which had formerly been described as "coma" in Syrian gold hamsters, rhesus monkeys and mice correspond rather to a stupor if the histological basis is also taken into consideration. The revision of our previous opinion has been initiated by the fact that in a comatous state the patient is insensitive to pain and does not respond to stimuli, whereas the experimental monkeys, sheep, hamsters, mice and rabbits although lying prostrate in the terminal stage were still responsive to pain stimuli, moving their extremities which otherwise appeared completely paralysed.

The haemorrhages in the gastric mucosa frequently found at postmortem examination, could well be attributed to the microscopic lesions in the tuber cinereum and the lateral hypothalamic region, whereas the "bladder paralysis" almost invariably present in our cases may be explained by the lesions in the paraventricular and supraoptic nuclei.

Like in natural rabies, the symptoms indicating injury of the hypothalamus were manifested in a well defined sequence also in the experimental disease. Although the histological follow-up of the process was rather difficult, it was clearly shown that severe lesions initially appeared in the anterior part of the hypothalamus, whereas towards the end of the course the posterior part was more affected. This accords well with the experimental observation that animals spontaneously died of infection with fixed virus, showed more severe changes in the posterior than in the anterior hypothalamus, and the same was found in histological studies on the brains of foxes succumbed with "natural" rabies (KARASSZON, 1973).

It follows that rabies virus does not affect all hypothalamic nuclei simultaneously, but in a well defineable sequence and the clinical symptoms follow the same course so that they indicate the route along which the virus is spreading. The early symptoms are essentially parasympathetic excitation phenomena, such as myosis, enophthalmus, vasodilatation, bradycardia, bronchial constriction, oesophageal spasm, gastrointestinal haemorrhages, erection etc. The further spread of the virus is towards the lateral nucleus, then to cell groups of the central hypothalamic region. After the involvement of nerve cells in the ventromedial region, the virus spreads caudally, affecting the posterior hypothalamic fields, which results in a score of sympathetic excitation phenomena, such as vasoconstriction, bronchodilatation, oesophageal spasmolysis, retention of urine, dilatation of the pupil etc.

The present findings also throw light on the problem of the "elective

involvement" of the hypothalamus. On the basis of histological evidence, the hypothalamus, as the regulator of all vegetative functions, can be held responsible for some characteristic symptoms of rabies and the gradual involvement of the hypothalamic nuclei accounts for the succession of the symptoms. The conflicting opinions on the "elective" involvement of the hypothalamus may have been due to the fact that as a rule no systematic study of the entire hypothalamus was made, but only preparations obtained from either the anterior or the posterior part were examined. Reliable results can only be obtained if the histological follow-up is based on the trend of virus spread, *viz.*, in the initial stage the anterior part, whereas in the terminal stage the posterior part of the hypothalamus is studied most thoroughly.

The present findings offer no explanation for the causes of the sequence in which the hypothalamic nuclei become involved. It remains to be decided whether the sequence depends on the variable sensitivity of the nuclei or simply signifies the trend of virus spread. If the latter assumption is true, the virus might well be regarded as a marker of the intricate interrelationships between hypothalamic nuclei.

In view of the histological findings, it cannot be excluded that the behavioural changes arisen in consequence of intracerebral inoculation with fixed rabies virus may also be associated with hypothalamic lesions. It is known that the hypothalamus is the integrative centre of the somatic and visceral components of emotionalm anifestations so that its pathological alteration brings about disintegration and in co-ordination of behavioural reactions. We are aware that investigations into emotional balance and behaviour cannot be centered on the hypothalamus and should be extended at least to the limbic system, as we had done in the case of rabid foxes. However, such complementary examinations would have been beyond the scope of the present study and so were investigations into disorders of the hypothalamic neurosecretory function. It is even difficult to harmonize the histological lesions with the clinical features observed in the present experiments. Furthermore, it is known that even in electric stimulation experiments, particular response to a single focal point is not conclusive in itself. Consideration should also be elicit parasympathetic excitation phenomena, but may also enhance the sympathetic influence. The development of isolated responses to stimulation of separate fields depends on various factors, such as the intensity of the stimulus, individual predisposition etc. (HESS, 1954). In the present case, the symptoms were due to inflammatory-degenerative changes of entire regions rather than to isolated functional disorders of one or another hypothalamic nucleus. The sequence of the manifold symptoms stems from an intricate multitude of reactions which are difficult to follow up morphologically whence the conclusions we have drawn may well be judged as arbitrary. We nevertheless believe that the histological evidence presented in this study is sufficient

to show that many symptoms of rabies are directly related with histopathological lesions of the hypothalamus, the organ primarily responsible for the maintenance of "homoeostasis" and that this approach may open up new scopes for investigations into the pathogenesis of rabies.

## Summary

Histopathological studies on the central nervous system of experimental animals (rhesus monkeys, sheep, albino mice, Syrian gold hamsters and rabbits) spontaneously died or killed in the terminal stage of disease, after intracerebral inoculation with Hőgyes' fixed strain of rabies virus showed that acute encephalitic lesions were always present in the hypothalamus too. Glia cell and mesenchymal cell reactions and degenerative changes of the neurons were usually more severe in the hypothalamus than in other brain areas.

In the early stage of the disease the anterior part of the hypothalamus, whereas in the advanced stage its posterior part was more affected.

All disorders of the autonomous nervous system known as the characteristic symptoms of rabies are attributed to the histopathologic lesions found in the hypothalamus and the sequence of the symptoms is explained by the successive involvement of the hypothalamic nuclei.

The histological evidence presented in this paper throws light on the much disputed problem of "elective involvement" of the hypothalamus and the use of the strictly neurotropic fixed virus in experimental neurology is suggested as an entirely new approach.

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Address of the author: Dr. Dénes Karasszon, 1966 Budapest, Gyáli út 2—6, Hungary

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# MASS INCIDENCE OF ENTROPION IN LAMBS

By

J. ZÁJER, T. KÉGL and K. SOMOGYVÁRI

Department and Clinic of Surgery and Ophthalmology (Head: Prof. A. B. KOVÁCS), University of Veterinary Science, Budapest

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Entropion, *i.e.*, inversion of the margin of the eyelid may reach such a degree that the eyelashes and lid skin touch the eyeball.

The condition occurs in all species of animals but, according to KÓMÁR and SZUTTER (1968), especially often in the dog and sheep. It may be congenital or acquired later in life.

Entropion in sheep has been studied by many authors (BAKER, 1920; CROWLEY and MCGLOUGHLIN, 1963; LITTLEJOHN, 1954; MCMANUS, 1960; SKIDMORE, 1928; WARWICK and BERRY, 1962; WRIGHT and FORMSTON, 1943); in Hungary, it was investigated by HÁMORI (1965). LITTLEJOHN (1954) reported a 50% incidence of entropion among newborn lambs in certain sheep flocks and MCMANUS (1960) found it to occur in 80% of sheep in a flock studied.

The problem of the heredity of entropion has been discussed in numerous publications. LITTLEJOHN (1954) believes that a predisposition is hereditary, the disease itself not, while others advocate the heredity of the condition itself by an as yet unknown mechanism.

If the entropion is not treated, conjunctivitis and keratitis may develop. The consequent corneal opacity and ulceration may lead to perforation and concomitant panophthalmitis, phthisis bulbi and blindness.

Of the different forms of acquired entropion spasmodic entropion is the most frequent. It is caused by lasting spasms of the palpebral muscle, which arises in the course of a painful conjunctivitis. Spastic entropion is refractory to various kinds of treatment, such as antibiotics and cortisone preparations, and even to ultrasound.

The cicatricial entropion a rare condition, may occur in all animal species. It arises either as a consequence of scar tissue formed after a mechanical injury of the lids (tarsus) or conjunctiva, or as a sequel to chronic conjunctivitis.

The third form, entropion bulbare, is due to a congenital smallness or absence of the eyeball or, probably, to a decrease in the volume of retrobulbar tissue.

## Experimental

Mass incidence of entropion among suckling lambs has been observed every year since 1967 in a district of Western Hungary. As the condition frequently caused blindness, with consequent emaciation and even death, economic losses were considerable in the affected flocks.

Incidence. In the 12 sheep flocks studied, generally 3—10% of the lambs had entropion. Exceptionally, incidence was as high as 20% and in one flock it even reached 70% in 1968; the right eye alone was involved in 40—60% of the cases, the left eye in 20%, and both eyes were affected in 20—40%.

The frequency of occurrence was generally unrelated to sex. The symptoms appeared at 10—20, occasionally one or two days of age. We have never observed the condition in newborn lambs or its first appearance above six weeks of age.

In merino breeds entropion is so rare that even the shepherds may not know it. Mass incidence of the condition was first observed after the crossing of merino ewes with Ascanian, Grosny, Caucasian and precoce rams for improvement of wool quality. It is important that neither ewes nor breeder rams showed a wrong position of the eyelids. Another important fact is that the crosses have a conspicuously wrinkled skin over the entire body.

The occurrence of entropion was limited to the lower eyelid; it was never found in the upper one. About 60—70% of the diseased lambs died, above all those with bilaterial involvement of the eye. Loss of condition owing to the eye disease resulted in a marked growth retardation among the survivors.

## Aetiology

Initially the entropion was believed to be congenital, for the frequent occurrence of a congenital, probably hereditary, inversion of the eyelid among sheep has been reported from all parts of the world since the 'twenties (BAKER, 1920; CROWLEY and MCGLOUGHLIN, 1963; LITTLEJOHN, 1954; MCMANUS, 1960; SKIDMORE, 1928; WARWICK and BERRY, 1962; WRIGHT and FORMSTON, 1943). Since, however, the lambs in the present study did not show any abnormality of the eyelid at birth, their illness should be attributed to reasons other than congenital deformity.

On analyzing the condition from the point of view of the pathogenesis of acquired entropion, the spasmodic form was suspected first, because the inversion ceased for the duration of local anaesthesia produced by a 1% Pantocain solution. Turning-inward nevertheless persisted unless the lid was drawn down with the finger at the onset of anaesthesia.

Of the several week old lambs 70—80% showed conjunctivitis. The condition was usually very mild, but in certain cases it was manifested by marked

lacrimation, catarrhal exudation, reddening and swelling of the conjunctiva and, occasionally, by swelling of the eyelid. Turning inward of the margin of the eyelid was chiefly associated with the severe cases of conjunctivitis.

Investigations into the cause(s) of the joint occurrence of conjunctivitis and entropion also included a study of the management and feeding conditions, but no gross failure or error was found in any farm. Bacteriological examinations of exudation from the conjunctival sac did not show the presence of any specific pathogenic agent.

Consideration was therefore given to the possible responsibility of a structural abnormality of the eyelid in the crosses. Pathohistological examinations carried out with the usual techniques failed to show any such abnormality and thus could not account for the cause(s) of entropion.

It was found on inspection of the crosses that the characteristic wrinkles were very pronounced also over the eyelids so that on closing of the eye the folds of the lower lid were bulging towards the edge, causing the lashes to point towards the eyeball and occasionally getting trapped below the upper lid of the winking eye. In certain cases the skin fold below the margin of the lower lid was so thick as to held the lashes permanently in an upright position, making them to touch the cornea even when the eye was open. Irritation of the eyeball by the lashes resulted in a painful conjunctivitis and keratitis which soon brought about the spasmodie entropion. The irritation of the conjunctiva and cornea elicited reflectorically the spasm of the orbicular muscle and the simultaneous, also reflectoric, spasm of the retractor muscle caused enophthalmus. Thus the eyelid lost part of its support and the lower lid, having a less developed tarsus than the upper one, turned inward in consequence of the blepharospasm.

The condition resembled the entropion caused in the human infant by epiblepharon (VELHAGEN, 1960) which may normalize later in life. As already mentioned in the foregoing, no entropion was found among lambs above six weeks of age and milder cases healed without any therapeutic intervention.

Thus the entropion of the hybrid lambs, characterized by a marked folding of the skin all over the body, is essentially due to the presence of cutaneous folds on the eyelids. Not the entropion itself is therefore inherited, only the predisposing factor, *viz.*, the loose texture of the palpebral subcutaneous connective tissue.

*Prognosis.* This is good if the therapeutic innervention is made in the early stage and the case is uncomplicated, it is dubious if purulent keratitis, corneal ulcer or pannus is present and bad if there is panophthalmitis.

## Therapy

Among the methods proposed for conservative treatment of entropion, repeated anaesthesia of the conjunctival sac is not practicable as a flock therapy. Injection of sterile paraffin oil of body temperature beneath the palpebral skin (KÓMÁR and SZUTTER, 1968) had an unfavourable effect on dogs in our hands, *viz.*, marked induration often developed at the site of the injection, leading to elephantiasis of the eyelid. Fixing of the eyelid in normal position by mattress suture (BLASKOVICS, 1945) was tried on three sick lambs, but infection of the subcutaneous connective tissue through the stab canal occurred in all three cases, resulting in phlegmon and abscess formation. Although the sutures ensured a proper fixing of the lid, they had to be removed prior to healing to prevent further suppuration.

In view of the above therapeutic failures, surgical repair was contemplated. The great number of diseased lambs did not, however, permit the application of refined techniques and time- and facility-requiring procedures.

As to the therapy of spasmodic entropion in humans, VELHAGEN (1960) proposed the subcutaneous administration of 1 ml 70% ethanol into the inversed lid, in order to lyse the spasm by durable weakening of the orbicular muscle. The same procedure was employed with success for lambs and after the initial trials, several thousands of young animals have been treated with it for entropion since 1970. A thin hypodermic needle is used for subcutaneous administration of the ethanol so that the needle's tip should be 1 cm below the median line of the lower palpebral edge. The exact position of the needle was ascertained by moving, and subsequently 0.75—1.5 ml ethanol was injected, depending on the age of the lamb and the severity of the entropion. The mechanical effect of the injected ethanol immediately results in turning-out of the inverted lid and no recurrence is likely to occur during the next days, after which the entropion heals without leaving any trace. The slight swelling caused by the injection disappears in about 10 days; a small, painless intumescence may infrequently remain, not causing any complication. The intervention is rapid, simple and spares the animals.

Initially solutions of antibiotic (chloramphenicol, aureomycin, *etc.*) were dropped into the ethanol-treated eye every day for one week. Although the antibiotic treatment was omitted two years ago, the therapeutic results have been as good as before. Corneal opacity, occasionally occurring after the acute symptoms had subsided, is treated by dropping a 5% Dionin solution into the eye. An uncomplicated healing can be achieved in most cases, but scar tissue, of course, remains if there had been an ulceration.

Early treatment is imperative, because it abbreviates the healing period and ensures the preservation of corneal transparency. It is therefore important that flock attendants should pick out and mark fresh cases of entropion daily.

The veterinarian in charge of the flock therapy should perform the injection treatment three times, but at least twice, a week, but a single inoculation is sufficient for each affected lamb; a second dose has never been required for healing. The daily application of eye drops should be performed by flock attendants.

Isolation of lambs with entropion is not necessary. This would greatly interfere with the daily working schedule of the attendants to whom the daily observation of the flock for the selection and marking of fresh cases is an extra duty anyway.

We believe that predisposition for entropion is no reason for giving up cross breeding for improved wool and mutton, because the eye disease can be controlled by appropriate therapeutic measures.

## Summary

Entropion of the lower eyelid has been observed since 1967 in 3—10% of lambs cross-bred from merino ewes and Ascanian, Grosny, Precoce or Caucasian rams. The eye disease was manifested between 10 and 20 days of age, and about 60—70% of the sick lamgs died in it.

Aetiological and therapeutic studies were carried out in 12 affected flocks. No congenital entropion was found and no specific pathogenic agent could be isolated from the diseased eyes. Pathohistological examinations revealed no structural abnormality of the eyelids. The cross-bred lambs, characterized by a marked folding of the skin over the entire body, developed a spasmodic entropion as a result of irritation of the cornea by the eyelashes, which was caused by turning-inward of the edge of the lower lid towards the eyeball by the mechanical effect of the cutaneous folds also present in the palpebral region. Thus the condition itself is not hereditary, only the loose texture of the palpebral subcutaneous connective tissue, which predisposes for entropion of the lower lid. Subcutaneous administration of 0.75—1.5 ml of 70% ethanol into the lower lid proved to be a simple, rapid and sparing therapeutic procedure and has since been employed with success for the control of entropion in several thousands of cross-bred lambs.

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Address of the senior author: Dr. József Zájer, H-1400 Budapest, P.O.B. 2, Hungary

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# EFFECT OF DIETARY NITROGEN LEVEL ON ELECTROLYTE AND WATER METABOLISM IN SHEEP

## I. $K^+$ METABOLISM

By

B. JUHÁSZ, B. SZEGEDI and M. KERESZTES

Department of Physiology (B. JUHÁSZ), Institute for Animal Production Budapest, Hungary

(Received March 7, 1974)

**The urea taken up in the feed is broken down in the rumen by the urease enzyme, consequently, the ammonia content of the ruminal fluid rises. According to VISEK (1968), the $NH_3 \rightleftharpoons [NH_4]^+$ concentration is influenced by the actual pH of the rumen contents.**

The present experiments were planned on the basis of earlier studies on changes in the water and alkali ($Na^+$, $K^+$) metabolism, and saliva and urine production in sheep fed different doses of urea (range: 0.4—0.7 g/kg). It was found that the secretion rate of saliva decreased after the intake of urea. Blood ammonia concentrations rose immediately, whereas the blood urea level rose with a slight delay. The rise of ure concentration in the blood was followed by its rise in the saliva; the quantity of urea excreted per unit time, nevertheless, fell considerably already during the first hour, owing to a significant decrease in the secretion rate. It follows from these observations that both the composition of, and the secretion rate for, saliva are influenced by the quantity of ingested nitrogen. If N-uptake is high, less saliva is produced and the total quantity of urea recycling with saliva into the rumen is reduced for a certain time. The factors responsible for this phenomenon should probably be sought in the rumen. The amount of sodium secreted with the saliva fell to one sixth already during the first hour, then it rose until reaching the initial level in the seventh hour after feeding. Potassium decreased to a similar degree, but did not return to normal in seven hours. The abrupt decrease of saliva secretion was accompanied by a gradual increase in diuresis. The concentration of $Na^+$ did not change in the serum, but the $K^+$ level decreased significantly. The amount of $Na^+$ excreted in the urine increased slightly in the third hour, then it tended to decrease. $K^+$ excretion rose notably in the first hour, but returned to normal after five hours. Accordingly, the salivary $Na^+/K^+$ quotient increased, whereas the urinary quotient ($K^+/Na^+$) decreased markedly (JUHÁSZ and SZEGEDI, 1968, 1969).

The results of earlier studies on toxicity of urea, on the rate of its decomposition in the rumen under different conditions and on the effects of ammonia on the metabolism of inorganic substances (SZEGEDI and JUHÁSZ, 1971) were also taken into consideration when the distribution of $K^+$ between blood plasma and erythrocytes was examined at rising concentrations of blood ammonia.

## Materials and Methods

### *Experimental animals*

Sixteen Hungarian Merino sheep, weighing 25—30 kg, were used. Before the experiment the animals were provided with a Jarret ruminal fistula. The animals received a commercial mixed fodder diet (300 g/day) supplemented with vitamins and inorganic salts, plus medium-quality meadow hay and

drinking water *ad libitum*. Twelve hours before the beginning of the experiment feeding was stopped, and only drinking water was provided, subsequently 0.6 or 0.8 g/kg body weight (bwt) levels' of urea were administered directly into the rumen through the Jarret fistula, mixed in 100 g fodder plus 400 ml water. The control animals received the same diet, without urea. Samples of blood and ruminal fluid were taken from both control and urea-treated animals from the jugular vein and through the Jarret fistula, respectively, at zero time and 1.5, 3, 4.5, 6 and 8 hours after feed consumption.

*Analytical methods*

The ammonia contents of rumen fluid and blood were determined by the microdiffusion method (JUHÁSZ and SZEGEDI, 1958). The haematocrit value of the blood was also measured.

The experiments were performed with $^{42}K$. The isotope was administered to the animals as $^{42}KCl$ (1 mCi/ml) isotonic solution by intravenous route 12 hours before the experiment. The dose was so selected that activity of the samples should be about 100 $\mu$Ci at the beginning of the experiment. The activity of the samples (blood, plasma, erythrocytes) was determined using a well-type scintillation detector and a GAMMA NK 108 counter.

*Mathematical evaluation*

The experimental data of the two groups (controls, and urea-treated animals) *viz.*, blood ammonia concentration, changes in the $^{42}K$ activity in blood, plasma and erythrocytes, distribution of the isotope between plasma and cells, were calculated and expressed as per cent the initial (0 hour) value. The correlation between blood ammonia level and percentual changes of distribution (k) was established from all measured values. Calculations were also made in the linear and exponential relations.

The *blood ammonia* concentration (Fig. 1) of the controls rose slightly 1.5 hours after feed consumption, then fell, and rose slightly again during the 6th hour; in urea-treated animals it increased markedly until the 3rd hour, then fell gradually but still remained above the initial value at 8 hours.

The $^{42}K$ activity of *whole blood* (Fig. 2) of controls increased until 4.5 hours, whereafter it decreased; in the urea-treated animals it fell until the 3rd hour, then rose.

The activity of the *plasma* (Fig. 3) of controls tended to decrease until the 3rd hour, then it temporarily rose, and fell again during the 6th hour. The plasma $^{42}K$ activity of urea-treated animals also fell until the 3rd hour and afterwards rose gradually until the end of the experiment. The rise coincided with the fall of the ammonia level. The decrease of plasma $^{42}K$ activity

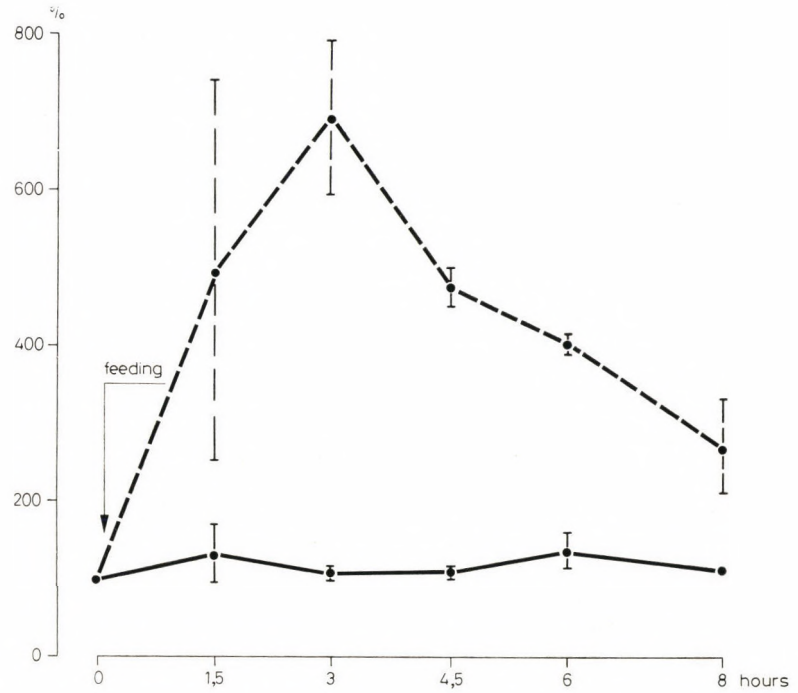


*Fig. 1.* Dynamics of blood ammonia after urea treatment. ——— Control; - - - - - - urea i.r.

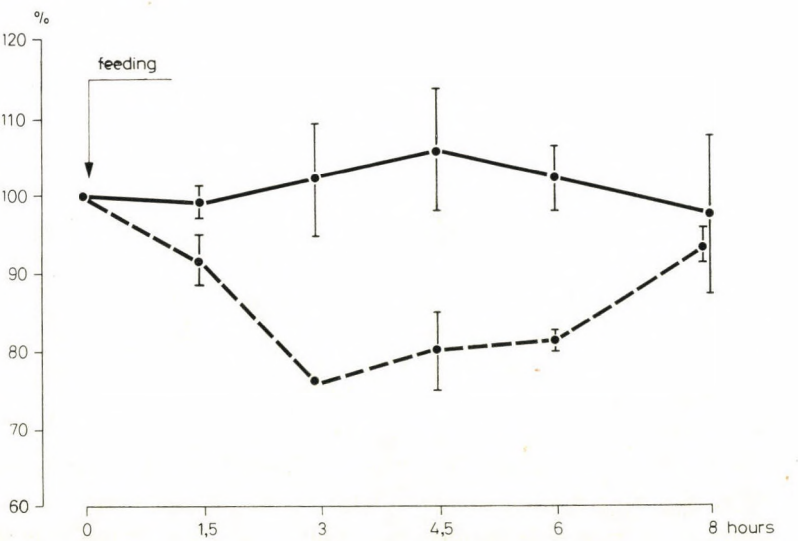


*Fig. 2.* Changes of $^{42}K$ activity in whole blood. ——— Control; - - - - - - urea i.r.

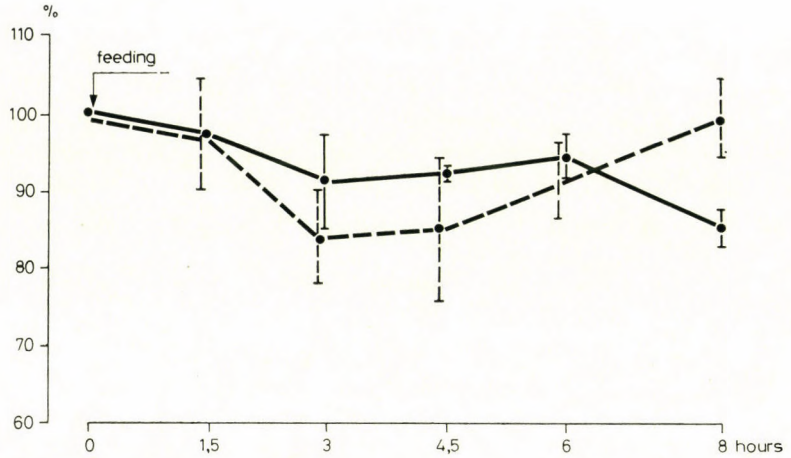


*Fig. 3.* Changes of $^{42}K$ activity of blood plasma. ——— Control; - - - - - - urea i.r.

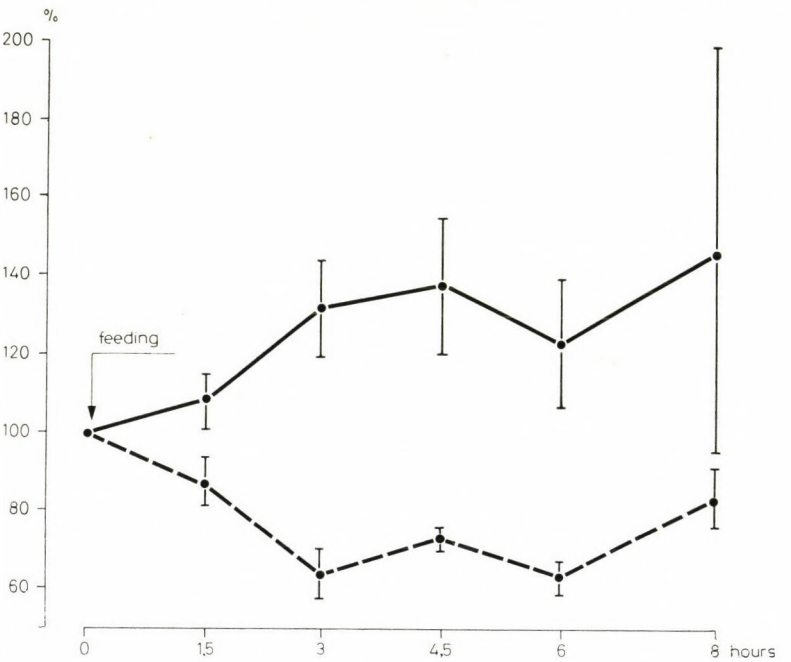


*Fig. 4.* Changes of $^{42}K$ activity of erythrocytes. ——— Control; - - - - - - urea i.r.

occurred in both cases when the ammonia concentration was at peak in the blood.

The $^{42}K$ activity of the *erythrocytes* (Fig. 4) of controls rose slowly but consistently, showing a temporary fall only during the 6th hour; in urea-treated animals it tended to fall and remained at a low level from the third hour until it began to increase after 6 hours.

## Results

The *distribution* coefficient (Fig. 5) *viz.*, the distribution of $^{42}K$ between erythrocytes and plasma of the controls followed the course of the curve for the $^{42}K$ activity of the erythrocytes (Fig. 4); in urea-treated animals it showed a falling tendency throughout the entire period of the experiment. The data of the experiments are summarized in Table I.

**Table I**

Blood ammonia level, $^{42}K$ activities in blood, plasma and erythrocytes, and the distribution coefficient of $^{42}K$ between plasma and red cells expressed in percentage of the initial levels

| Samples time hours | Blood ammonia level | | Blod | | $^{42}K$ activity in | | | | Distribution coefficient (k) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Plasma | | Erythrocytes | | | |
| | % | S* | % | S* | % | S* | % | S* | % | S* |
| Control animals | | | | | | | | | | |
| 0 | 100 | — | 100 | — | 100 | — | 100 | — | 100 | — |
| 1.5 | 135 | ±35 | 99 | ±2 | 97 | ±4 | 108 | ±7 | 112 | ±11 |
| 3 | 107 | ±3 | 102 | ±7 | 91 | ±6 | 132 | ±12 | 145 | ±12 |
| 4.5 | 111 | ±4 | 105 | ±8 | 92 | ±1 | 138 | ±17 | 149 | ±17 |
| 6 | 140 | ±21 | 102 | ±4 | 94 | ±3 | 123 | ±16 | 133 | ±22 |
| 8 | 115 | ±0 | 97 | ±10 | 85 | ±2 | 147 | ±52 | 170 | ±56 |
| Urea-treated animals | | | | | | | | | | |
| 0 | 100 | — | 100 | — | 100 | — | 100 | — | 100 | — |
| 1.5 | 496 | ±247 | 92 | ±3 | 97 | ±7 | 87 | ±6 | 91 | ±11 |
| 3 | 695 | ±97 | 76 | ±0 | 84 | ±6 | 64 | ±6 | 78 | ±12 |
| 4.5 | 477 | ±22 | 80 | ±5 | 85 | ±9 | 73 | ±2 | 89 | ±10 |
| 6 | 406 | ±4 | 81 | ±1 | 91 | ±5 | 63 | ±4 | 69 | ±5 |
| 8 | 274 | ±61 | 93 | ±2 | 99 | ±5 | 84 | ±7 | 86 | ±11 |

S*, standard deviation

## Discussion

The $K^+$ contents of blood plasma and erythrocytes differ markedly. Kolb (1967) gives the concentrations of $K^+$ in sheep blood, plasma and erythrocytes as 35, 18 and 80 mg/100 ml, respectively. The unequal concentrations of $K^+$ are related to the extra- and intracellular distribution of cations and anions, which applies particularly to $K^+$ and $Na^+$. The passage of $K^+$ from the extracellular fluid into the intracellular space is an energy-requiring transport process. Release of $K^+$ from the cells takes place by dif-

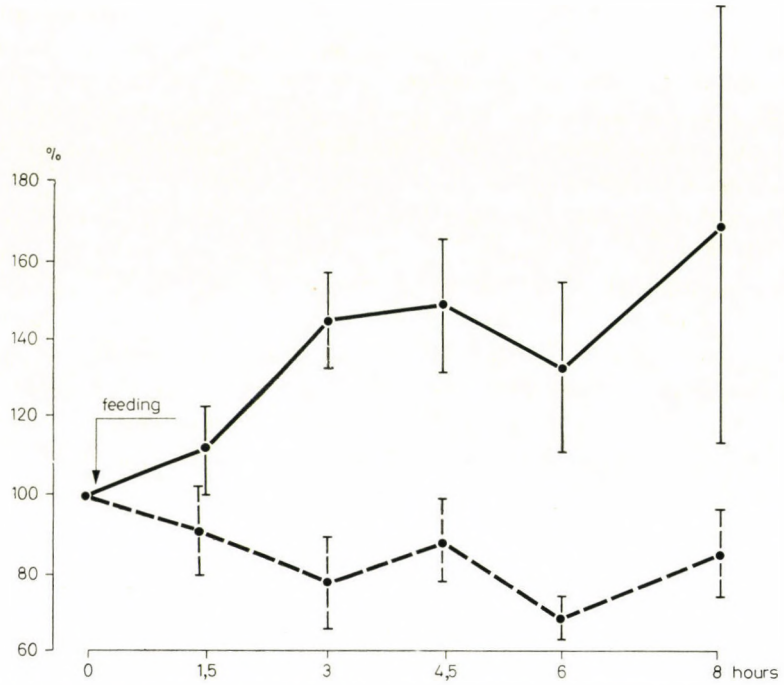


*Fig. 5.* Dynamics in the distribution (k) of K in the blood after urea treatment. ——— Control; - - - - - - urea i.r.

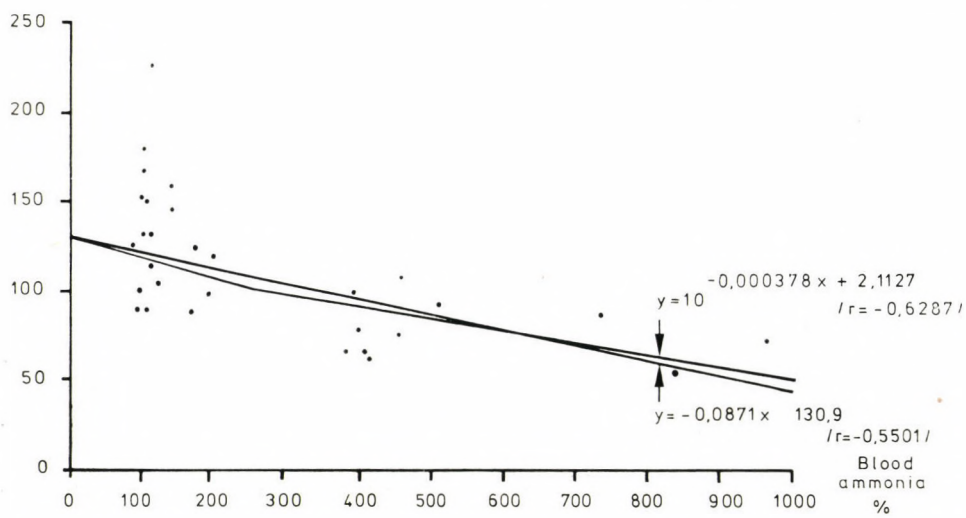


*Fig. 6.* Correlation between changes of blood ammonia level (%) and $^{42}K$ distribution coefficient

fusion. The flow of $Na^+$ between the two systems requires reverse conditions of energy. If the energy-producing processes of erythrocytes are paralyzed with monoiodoacetic acid, the transport of $K^+$ across the cell membrane will decrease whereas the release of $K^+$ from the cell will continue as usually, so that the $K^+$ concentrations of the two systems become equilibrated in due course (WEBB, 1966).

In the control animals, absorption of a greater amount of $K^+$ from the feed resulted in a rise of $K^+$ in the blood after feeding (Fig. 2). The rise was due to the increased $K^+$ content of erythrocytes (Fig. 4), whereas the $^{42}K$ activity (Fig. 3) and the $K^+$ content of the plasma fell. This diminution is related to renal function in ruminants. After food intake, the kidney eliminates more $K^+$ than $Na^+$, thus, simultaneously with a temporary rise of intracellular $K^+$ and fall of extracellular $K^+$, reserves (JUHÁSZ and SZEGEDI, 1958, 1969) which begins to normalize after 4.5 hours. At the same time, the percentual distribution of $^{42}K$ between erythrocytes and plasma fluctuated considerably (Fig. 5).

In earlier studies (SZEGEDI and JUHÁSZ, 1971) the rates of decomposition and utilization of the administered urea varied individually. Blood ammonia rose to a lower level in those animals which utilized the urea well, compared to those which either utilized it less efficiently or were given the higher dose (Fig. 1).

In sheep given urea, the $^{42}K$ activity of the plasma decreased more markedly than in the controls (Fig. 3) and there was a simultaneous reduction in the $^{42}K$ activity of the whole blood (Fig. 2). Unlike controls, the erythrocytic $^{42}K$ activity also fell (Fig. 4). It was shown earlier (SZEGEDI and JUHÁSZ, 1971) that 4 hours after the administration of urea, its urinary excretion increased considerably and increased amounts of $K^+$ were eliminated. This finding can account for the marked decrease of plasma $^{42}K$ activity (Fig. 3), but cannot explain the activity decrease of $^{42}K$ in the erythrocytes (Fig. 4). Since the haematocrit value changed to an equally moderate degree in both the control and urea-treated animals, it is concluded that the fall of the erythrocytic $^{42}K$ activity does not necessarily mean a decrease of the total ion concentration, nor of water intake. It seems that the rise of blood ammonia level inhibits the transport of $K^+$ from the extracellular space into the cells, and promotes the release of $K^+$ from the cells by diffusion.

It was found that, owing to the inhibitory action of ammonia, a certain correlation exists between the rise of blood ammonia level and the distribution quotient of $K^+$. The higher the blood ammonia level, the greater the inhibition of $K^+$-transport into the erythrocytes. An equation of regression was formulated to express this correlation between blood ammonia level and distribution quotient (Fig. 6). When calculated with linear values, the correlation coefficient was found to be $r = 0.5501$. Since the graphical presentation and compari-

son of paired values suggested the correlation to be exponential rather than linear, the values of the blood ammonia concentration were plotted against the logarithms of the distribution quotient. The correlation coefficient of the regression thus calculated was $r = 0.6287$.

The precise mechanism of this phenomenon remains to be clarified by further *in vitro* studies. This will include the influence of various concentrations of ammonia on $K^+$ transport and comparison of the inhibitory effect to that of monoiodo acetic acid.

We are aware that $Na^+$ and water metabolism are also involved in the process, and experiments with radiosodium ($^{24}Na$) are in progress to obtain more information on the problem.


## Summary

The distribution of $^{42}K$ between whole blood, plasma and erythrocytes was studied in sheep after intraruminal administration of 0.6—0.8 g/kg body weight urea, at rising concentrations of blood ammonia.

The ammonia content of rumen fluid and blood and haematocrit value were determined. The $^{42}K$ isotope was administered i.v. The activities of blood, plasma and erythrocytes were determined at zero time and 1.5, 3, 4.5, 6 and 8 hours after administration of urea.

In sheep given urea, the $^{42}K$ activity of the plasma decreased more markedly than in the controls and there was a simultaneous reduction in the $^{42}K$ activity of whole blood. Unlike controls, the erythrocytic $^{42}K$ activity also fell.

It seems that the rise of the blood ammonia level inhibits the transport of $K^+$ from the extracellular space into the cells, and promotes the release of $K^+$ from the cells by diffusion.

It was found that, owing to the inhibitory action of ammonia, a certain correlation exists between the rise of blood ammonia level and the distribution quotient for $K^+$. The higher the blood ammonia level, the greater the inhibition of $K^+$ transport into erythrocytes. An equation of regression was formulated to express the correlation between blood ammonia level and distribution quotient


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Address of the senior author: Dr. Balázs JUHÁSZ, 1024 Budapest, Kitaibel Pál u. 4, Hungary

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# CYTOTOXICITY STUDIES ON T-2 FUSARIOTOXIN

By

L. BODON and L. ZÖLDÁG

Veterinary Medical Research Institute (Director: D. DERZSY),
Hungarian Academy of Sciences, Budapest

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Many strains of the genus *Fusarium* have metabolic products toxic for both man and farm animals. The main toxic products of *Fusarium* fungi are the oestrogenic zearalenone (F-2), 12, 13-spiroepoxysesquiterpenoid derivatives (toxin T-2, diacetoxyscirpenol, nivalenol, fusarenon-X, solaniol, etc.) and butenolide.

Apart from chemical procedures (thin-layer chromatography, ultraviolet and infrared spectrophotometry, gas chromatography, etc.), biological assays are still important means of toxin demonstration, because many toxins cannot be precisely identified by analytical methods alone.

Interest has recently been focussed on the sesquiterpenoids, compounds of trichothecane structure, in connection with aetiological studies on the fusariotoxicosis of domestic animals. The chemical assay of these toxins is hampered by the fact that they do not show fluorescence in ultraviolet light and as yet no specific reactants have found to them.

Several authors have studied them by cutaneous test (ELLIS and YATES, 1971; UENO et al., 1971a, b, c) and in tissue culture (UENO et al., 1972) and a useful assay based on a selective microorganism was also developed (BURMEISTER and HESSELTINE, 1970).

The present paper is a report on studies of the fusariotoxin T-2 in tissue culture.

According to literary data, T-2 is chiefly produced by certain strains of *Fusarium tricinctum* (BAMBURG et al., 1967), but it was also demonstrated with the species *F. solani*, *F. rigidiusculum* and *F. roseum*, *F. sporotrichioides* and *F. poae* (UENO et al., 1972b). It was several years earlier that the Russian author OLIFSON (1965) attributed the toxicity of the latter two species to toxic fungal products of sterane structure (sporo- and poaefusariogenine). According to the systematics of SNYDER and HANSEN (1940), *F. sporotrichioides* and *F. poae* are synonyms of *F. tricinctum*.

## Materials and Methods

*Toxin T-2.* A pure crystalline toxin, prepared in the Institute of Professor Yoshio UENO (Japan) was used throughout. To make a stock solution of 500 mcg/ml, the toxin was dissolved in slightly heated water.

*Tissue culture.* The permanent pig kidney epithelial cell line PK-15 was used throughout. After the outgrowth of the confluent monolayer, the growth medium was discarded and 7 ml of an 0.25% trypsin solution was added to the culture which was then incubated at 37 °C until cells began to detach. After incubation, 7 ml of M 199 medium, containing 10% colostrum-deprived calf serum and antibiotics (streptomycin, mycostatin) was added. The cell suspension, 14 ml in final volume, was divided into two equal parts: 7 ml were used for further passage, the rest was distributed to Leighton tubes each provided with a coverslip.

*Titration of toxin T-2 in tissue culture.* The T-2 stock solution (500 mcg/ml) was diluted in Earle's solution in tenfold serial steps, thus six dilutions from 50 mcg to 0.0005 mcg/ml were prepared. Four tube cultures were inoculated with each dilution, 0.1 ml/tube. The results were read under the microscope 1, 2, 3, 4, 21, 22, 23, 24 and 48 hours later.

*Staining procedure.* Two staining techniques were employed, haematoxylin-eosin and acridine orange (BODON, 1967). Preparations stained with acridine orange were extracted with 50% ethanol and stained again with haematoxylin and eosin.

*Rabbit cutaneous test.* This was performed with tenfold serial dilutions of the toxin in an isotonic phosphate buffer of pH 7.4, on white New-Zealand rabbits. From each dilution 0.01 ml was injected into the depilated skin.

## Results

*Tissue culture.* No cell changes occurred during the first four hours after inoculation. The cytotoxic effect began to develop after 21 hours. The time of appearance of cytotoxicity with the different dilutions can be seen in Table I.

**Table I**

Cytotoxicity assay of T-2 in tissue culture

| T-2 toxin dilutions, mcg/ml | Time of appearance of cytotoxic effect (hours after inoculation) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 21 | 24 | 28 |
| 5 | 0 | 0 | 0 | 0 | + | + | + |
| 0.5 | 0 | 0 | 0 | 0 | + | + | + |
| 0.05 | 0 | 0 | 0 | 0 | + | + | + |
| 0.005 | 0 | 0 | 0 | 0 | + | + | + |
| 0.0005 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.00005 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Control | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

0, no cytotoxic effect
+ distinct cytotoxic effect

*Description of the cytotoxic changes.* Both staining techniques employed revealed the following changes: the confluent monolayer became discontinuous, and the cells were linked with one another by elongated cytoplasmic bridges which showed a netlike pattern. There were many cell-free areas, in which the deteriorated cells became detached from the tube wall. The degree of changes was proportional to the degree of dilution; the last two dilutions of this series had no visible cytotoxic effect.

Cells at the margins of the cell-free areas were pyknotic, and smaller and longer than the control cells. The nucleus often fell apart to small globules (karyorrhexis), the cytoplasmic RNA content decreased and cytoplasmic vacuoles appeared.

*Rabbit cutaneous test.* This proved to be less sensitive than the tissue culture test. The dermatoxic effect was read 48 and 72 hours after inoculation. The maximum intradermal dose contained 5 mcg toxin, which elicited a distinct local cutaneous lesion. After 48 hours the skin exhibited reddening and was swollen and painful in an about 1 $cm^2$ area. Inoculation of 0.5 mcg toxin was followed by a similar, but milder, change and the further dilutions elicited no cutaneous reaction whatever.

## Discussion

Among the metabolic products of *Fusarium* fungi, toxin T-2 is one of the most dangerous cytotoxic agents. The present studies were aimed at the comparison of these two biological assays. Out of them the tissue culture test proved to be more sensitive and it seems to be suitable for the quantitative determination of the toxin. The correlation between toxin concentration and microscopically visible histological and cytotoxic changes was almost linear. The first cytotoxic changes appeared after 21 hours and progressed gradually, depending on the dose, until the end of the observation period at 48 hours.

Comparison of the results of cutaneous and tissue culture tests showed the latter to be 100 times more sensitive than the former. The minimal demonstrable amount of toxin was 0.005 mcg/tube in tissue culture, whereas in the cutaneous test 0.5 mcg elicited only a very slight reaction.

The role of toxin T-2 has up to now been considered in two animal diseases. In the United States, Ellis and Yates (1971) described the so-called fescue foot disease of cattle as a condition caused by the fungus *F. tricinctum*, growing on the tall fescue *(Festuca arundinacea)*. Apart from T-2, *F. tricinctum* produces butenolide and other T-2-like scirpenol derivatives. The disease occurs in animals turned out on pasture. Its main symptoms are lameness of the hind legs, in severe cases gangrene of the extremities, tail and ear, occasionally detachment of the horn of the foot. In Japan Ueno et al.

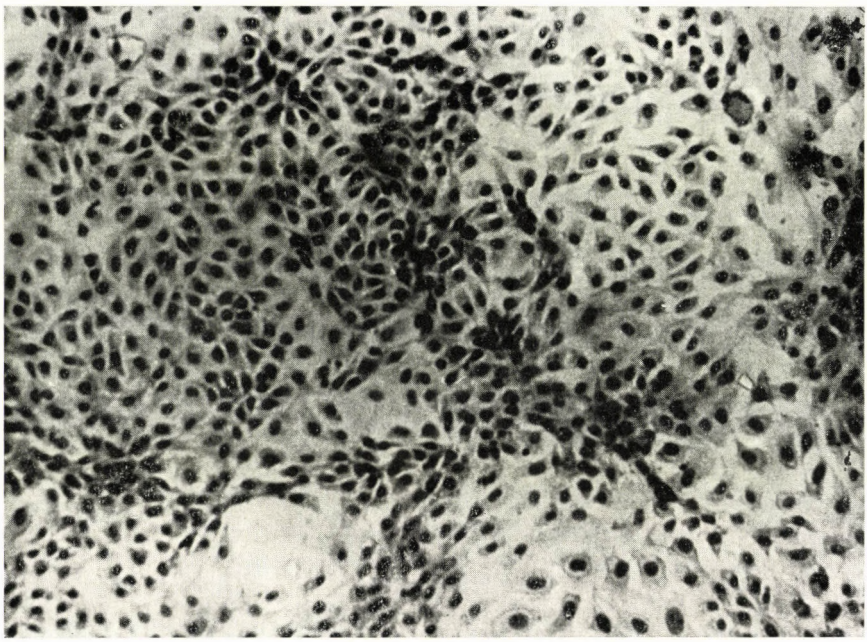

*Fig. 1.* Normal culture of the PK-15 kidney epithelial cell line. Control, ×60

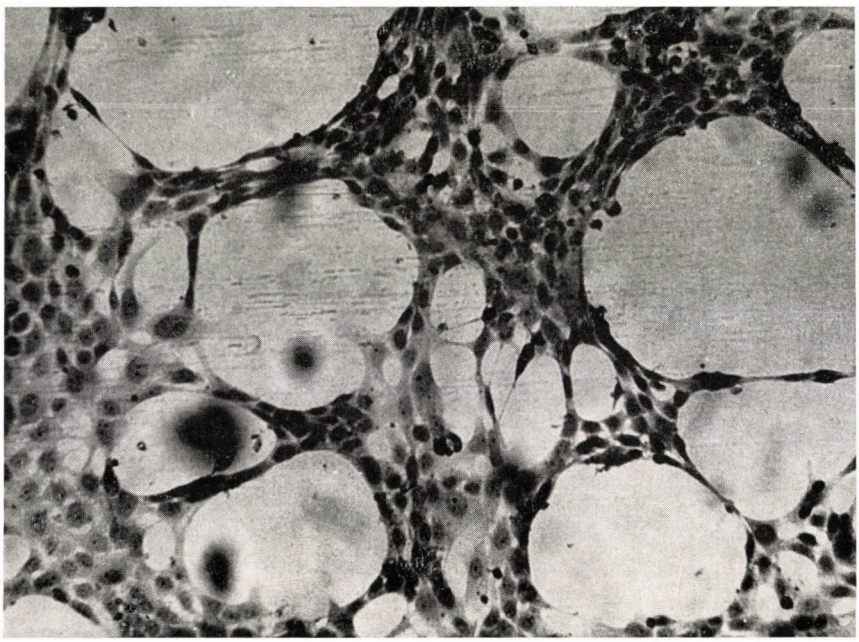

*Fig. 2.* PK-15 cell culture 21 hours after inoculation with T-2 toxin. ×60

(1972b) found in the course of aetiological studies on the hull poisoning of horses that it was caused by toxins of trichothecane structure, produced by *Fusarium* species infecting the bean. The symptoms of the acute or semi-acute toxicosis are disturbances of the central nervous system, convulsions, motoric disorders, compulsive movements, clonic convulsions of certain muscle groups, respiratory trouble and severe circulation disorder.

In Hungary, there has been no evidence of the aetiological role of T-2 and other scirpenol derivatives in the fusariotoxicosis of domestic animals, but since toxin-producing *Fusarium* fungi are widely spread in this country and toxin production is favoured by the climatic conditions, their probable responsibility cannot be excluded.

*Acknowledgement.* The authors are indebted to Professor Joshio UENO (Japan) for supplying the T-2 toxin preparation.

## Summary

The toxin T-2, produced by several species of *Fusarium* fungi was tested for cytotoxic effect in tissue culture and rabbit cutaneous test. The tissue culture proved to be very sensitive, *viz.*, as little amount of toxin as 0.005 mcg/tube already elicited well-defineable cytotoxic changes. Cell changes were read after staining with acridine orange and haematoxylin and eosin. The main changes were discontinuity of the monolayer, imparting a netlike appearance to the cell sheet, and pyknosis and rhexis of the nuclei. The tissue culture test is suitable for quantitative titration of the pure toxin. The lowest amount of toxin detectable by the rabbit cutaneous test was 0.5 mcg.

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Address of the authors: Dr. László BODON, Dr. László ZÖLDÁG, 1143 Budapest, Hungária krt. 21, Hungary

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# INFLUENZA INFECTION IN HORSE STOCKS CAUSED BY EQUINE A-1 AND HONG KONG SUBTYPE IN HUNGARY

By

J. ROMVÁRY, J. TANYI, Zsuzsa MÁTHÉ and E. FARKAS

Veterinary Medical Research Institute (Director: D. DERZSY), Hungarian Academy of Sciences, Budapest, Veterinary Institute (Director: R. HENGL) Debrecen, Veterinary Institute (Director: P. ÁLDÁSSY) Miskolc, and Hungarian Society of Microbiology, Budapest

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It was in 1955 that HELLER et al., gave account of a myxovirus infection first observed among horses, in the course of an outbreak in Sweden. The epidemic spread in the subsequent year toward Central Europe, *viz.* in the surroundings of Dresden and Prague. In Prague an influenza virus, the first one isolated from horses was recovered by SOVINOVÁ et al. (1958). This represented a new subtype of influenza A viruses, now classified as *A/equine/Prague/1/56 (Heq1Neq1)* or equine A-1 subtype of influenza virus. Serological evidence indicated the presence of this virus not only in certain European countries but also in other areas, indicating that horses in the United States as well as in Australia and Africa had been attacked by 1957. In certain areas the infection of horses was subclinical (USA, 1963, Great Britain, 1965, etc.).

A new severe outbreak was observed at Miami, Florida, early in 1963. An influenza-like disease was previously noticed in thoroughbreds imported from Argentina by air. A striking feature of this outbreak was that horses of all ages were equally affected. Subsequent laboratory investigations made by WADDELL et al. (1963) revealed that this outbreak was caused by an earlier unknown subtype of influenza A virus, different from equine A-1, now termed *A/equine/Miami/1/63(Heq2Neq2)*, the prototype strain of the subtype equine A-2. This epidemic spread very quickly amid significant severe clinical signs. In the epidemic areas about 80% of the horses were affected in the same year and the disease also shortly appeared in Canada. In Europe, this subtype was first recognized by its symptoms in Great Britain, then in other West-European countries as well.

Horses in 12 stocks were screened in Hungary by ROMVÁRY et al. in 1965 for HI antibodies to the influenza equine A-1 and equine A-2 subtype virus but antibodies were found only against subtype equine A-1. The incidence of these antibodies among the racehorses proved to be 48% while in the large state farms it was higher, *viz.*, 63—76%. In two closely connected stocks colts 2 and 3 years of age were tested. Of these, on an average, 49% had equine A-1 antibodies. At that time we failed to isolate the virus. The influenza equine A-2 subtype infection, with isolation of virus strains was firstly recognized in Hungary by ROMVÁRY et al. (1968).

## Materials and Methods

Properly pretreated nasal secretions taken from horses in acute stage of illness were inoculated into the amnioallantoic cavity of 9—10 days embryonated hen's eggs. The horse serum samples were pretreated with heat, trypsin and potassium periodate as directed by WHO circular in 1967. In the haemagglutination and haemagglutination inhibition tests chicken erythrocytes were applied, and 8 HA units of virus were used in the HI test. A recently isolated inhibitor-resistent strain of the influenza A Hong Kong subtype of human origin was used.

## Results

In May 1973, a mild acute respiratory disease in epidemic form had broken out in horse stocks in Hungary. In a state farm, among colts and adult horses, the clinical signs were: fever up to 40.2—40.6 °C, anorexia, deep, initially dry then wet coughing with serous-fibrinous nasal discharge. In the second passage of 9—10 days embryonated hen's eggs three haemagglutinating virus strains were isolated.

**Table I**

Results of cross HI tests

| Virus strains | 673 | 675 | 671 |
|---|---|---|---|
| | rabbit antisera to | | |
| | A/equine/Prague/1/56 | A/equine/Miami/1/63 | A/Hong Kong/1/68 |
| | viruses | | |
| A/equine/Prague/1/56(Heq1Neq1) | 1 : 256 | N | N |
| A/equine/Miami/1/63(Heq2Neq2) | N | 1 : 256 | N |
| A/Hong Kong/1/68(H3N2) | 1 : 4 | 1 : 8 | 1 : 512 |
| B251/1/73 | 1 : 128 | N | N |

The isolates (B251, B252 and M36) in the cross HI test carried out with rabbit antisera, proved to be equine A-1 subtype strains (see Table I).

In certain stocks paired blood samples were collected at an interval of 18—32 days from horses recovered from the disease. As compared to the first samples, the second samples yielded in the HI test more positive reactions and showed higher titres (see Table II).

Specific antibody could not be detected in either of the 10 sera tested in course of the first bleeding from each of stocks "S" and "B" but 21—32 days later 5 sera in each stock already reacted as positive. A rise in seropositivity rate was observed in three additional stocks. In stocks "Szv", "K" and "R" 93.3—100% of the horses gave positive HI reaction to equine A-1 virus.

For stocks where the age of horses had been known the distribution age-specific incidence of antibodies has been investigated (Table III). It is clearly seen that among the 1—5 years old horses these antibodies could be demonstrated in a higher percentage, *viz.*, 82% as than in the sera of 6—20 years old ones (62%). In all probability the colts till 5 years of age were more susceptible than the older horses.

From June 1973 till March 1974, in 19 horse stocks 286 blood samples had been collected and HI antibodies to influenza equine A-1 subtype virus

**Table II**

Testing of paired horse sera for the presence of HI antibodies to influenza equine A-1, equine A-2 and Hong Kong virus

| Stock | Dates of screening | Results of HI tests with | | |
|---|---|---|---|---|
| | | A/Prague/1/56 | A/Miami/1/63 | A/Hong Kong/1/68 |
| | | viruses | | |
| Szk | 24/6/73 | 3/10 | 3/10 | 5/10 |
| | 20/7/73 | 8/10 | 3/10 | 2/10 |
| S | 21/6/73 | 0/10 | 0/10 | 0/10 |
| | 12/7/73 | 5/10 | 0/10 | 0/10 |
| Nj | 25/6/73 | 4/10 | 1/10 | 3/10 |
| | 13/7/73 | 9/10 | 1/10 | 2/10 |
| D | 27/6/73 | 2/15 | 3/15 | 5/15 |
| | 23/7/73 | 7/10 | 3/10 | 3/10 |
| B | 18/6/73 | 0/10 | 1/10 | 2/10 |
| | 20/7/73 | 5/10 | 1/10 | 1/10 |
| Szv | 20/9/73 | 14/15 | 0/15 | 3/15 |
| K | 11/7/73 | 10/14 | 0/14 | 5/14 |
| | 22/2/74 | 9/9 | 1/9 | 0/9 |
| R | 11/6/73 | 2/10 | 4/10 | 1/10 |
| | 28/2/74 | 20/20 | 7/20 | 0/20 |

**Table III**

Distribution by age of horses seropositive to influenza equine A-1 virus in HI test

| Stock | Age in year | | | | | | | Total |
|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6–10 | 11–20 | |
| Szv | — | — | 4/5 | 5/5 | — | 4/4 | 1/1 | 14/15 |
| Ds | — | — | — | — | 0/1 | 3/6 | 6/8 | 9/15 |
| B | — | 0/1 | 2/3 | — | — | 5/8 | 1/3 | 8/15 |
| Szg | — | — | — | — | 1/2 | 3/10 | 1/3 | 5/15 |
| K | 2/2 | 3/4 | 1/1 | 2/4 | — | 1/3 | 1/4 | 10/18 |
| Ky | — | — | 4/4 | 4/4 | 3/3 | 6/9 | 2/2 | 19/22 |
| H | — | | — | 2/2 | 4/4 | 2/10 | 2/2 | 10/18 |
| R | — | — | — | — | — | 6/6 | 14/14 | 20/20 |
| | 2/2 | 3/5 | 11/13 | 13/15 | 8/10 | 30/56 | 28/37 | 95/138 |
| | | | 37/45 | | | 58/93 | | |
| | | | (82.2%) | | | (62.3%) | | (68.8%) |

could be detected, in 162 (56.6%) of these, mostly up to a serum dilution of 1 : 16—1 : 64. In each stock positive individuals had been in a variable rate.

In eight stocks the infection had been more intense, the seropositivity was superior, 87.6%, and by about 50% more blood samples proved to be positive up to 1 : 32—1 : 256 than in the other stocks.

The sera were screened for the presence of HI antibodies to influenza equine A-2 and only 51 (17.8%) of the sera collected from horses over 5 years of age gave positive reaction up to a serum dilution of 1 : 10—1 : 40. Sera of horses that had proved to be negative in the course of the first testing consistently remained negative.

The horse sera were tested simultaneously with a recently isolated inhibitor-resistant strain of the influenza A Hong Kong subtype of human origin. In the HI test 39 (13.6%) were positive up to a serum dilution of 1 : 10—1 : 160. It was evidenced that the Hong Kong antibodies could only be disclosed in sera collected till late September, 1973, that is, 5—8 months after an intensive wave of influenza epidemic in humans in the very same area.

## Discussion

In Hungary, the acute respiratory disease in horse stocks caused by influenza equine A-1 subtype virus broke out probably in May, 1973. Of the blood samples taken randomly in late June only few if any proved to be positive in the HI test, but in July the number of seropositive animals increased considerably. Some smaller stocks, first of all colt raising plants wherein in the course of a year horses had not been brought, remained free of influenza equine A-1 virus infection. After the epidemic the colts that had been transported to stocks recovered from influenza infection remained seronegative for months. In some stocks 60—75% of the horses had demonstrable antibody 3—4 weeks following infection. Further on, every individual might have infected in some stocks, what is supported by results of HI tests carried out in the second samples collected in stocks "R" and "K". The first appearance of HI antibodies in the second blood samples, or a significant titre increase was accepted as evidence of a recent infection. The same is also supported by high-grade infection of the horses 1—5 years of age. Together with a percentual increase in the infection rate within stock, the HI titres were increasing.

The mild respiratory syndrome accompanying influenza equine A-1 virus infection can hardly be differentiated from the rhinopneumonitis, but the isolation of the influenza equine A-1 virus strains, for the first time in Hungary, and the significant increase of antibodies in serum of convalescent horses had confirmed the right diagnosis.

The low-titre HI antibodies to influenza equine A-2 reference virus strain only in sera of over 5—6 years old horses should be considered residual

ones existing in animals that had got over a severe course of epizootic in 1968 caused by influenza equine A-2 subtype virus. In paired sera we could recognize an increase of antibodies to this virus in no case.

It has been shown that horses may be infected by the human Hong Kong subtype of influenza A virus and in their serum antibodies to this virus may be demonstrable. In accordance with this, anti-Hong Kong HI antibodies were demonstrated in 39 (20.6%) of the horse sera tested in June-September, 1973, probably due to mild infections during the intensive influenza epidemic in humans in the preceding winter. Further blood samples were collected from 156 horses in the winter 1973—1974, *i.e.*, one year after the Hong Kong epidemic. Specific antibodies were found even in none of the sera, indicating that the circulating antibodies had been fairly soon eliminated from the blood stream. In horse sera the specificity of HI antibodies to the Hong Kong virus has been confirmed by positive result of virus neutralization tests carried out in embryonated hen's eggs.

## Summary

Influenza virus strains of the equine A-1 subtype were isolated from nasal discharge of diseased horses, indicating mild acute respiratory symptoms in the course of an epizootic that broke out in Hungary late in May, 1973. In affected stocks of the 286 blood samples collected randomly, 56.6% proved to be positive in HI test, mostly up to a dilution of 1 : 16—1 : 64.

Residual antibodies to equine A-2 virus were detected only in 17.8% in serum of horses over 5—6 years of age, recovered from a severe equine A-2 epidemic in 1968.

39 (20.6%) of 189 blood samples gave positive reaction to Hong Kong virus 5 to 8 months subsequent to a human epidemic. These antibodies soon disappeared.

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Address of the senior author: Dr. József ROMVÁRY, 1143 Budapest, Hungária krt. 21, Hungary.

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*The Coccidia, Eimera, Isospora, Toxoplasma and Related Genera.* Edited by HAMMOND, D. M. and LONG, P. L. University Park Press, Baltimore, Butterworths, London, 1972, Pp. 482.

The volume, written by ten co-authors, deals with general problems of coccidiology and thus satisfies an old demand of coccidium specialists. It is printed on a paper of high quality and many light and electron micrographs, drawings and tables help the reader in profiting from its contents. Orientation in the text, which is divided into ten chapters, is facilitated by a subject index.

Chapter 1, written by N. D. LEVINE, deals on 22 pages with the systematics of coccidia and, above all, with the work of the scientists engaged in taxonomic problems. The classification of the subphylum *Apicomplexa* Levine, 1970, is interesting, new and well-organized, although it will take some time until the coccidiologists get accustomed to the unusual endings of certain taxon names. — Chapter 2, contributed by W. C. MARQUARDT, discusses on 20 pages the host and organ specificity of coccidia, disclosing much new information on tissue preference and intracellular localization of the stages.

In the third chapter D. M. HAMMOND describes, on 25 pages, the life cycle and developmental course of coccidia. The known and new details of the cycle are illustrated by many good pictures and the text gives answers to several open questions. It is not surprising that the author reverts to the developmental particularities of *Eimeria bovis* several times, because it was HAMMOND and co-workers who examined the process in greater detail by ingeniously planned experiments. They discovered that the developmental cycle of *E. bovis* includes grossly-visible giant schizonts, which had formerly been regarded as globidial of an unknown systematic position. Further investigations based on this finding have confirmed that several other species of *Eimeria* also have a globidial endogenous cycle. In view of the modern outlook on which the presentation of the chapter is based the reader feels surprised when mention is made of *E. gilruthi*. The implication that this globidiumlike body, occurring in sheep, were a coccidium is hypothetical and no systematic classification is possible on the basis of a single developmental stage found in the abomasum. A detailed description of *Isospora* development, sporogony and excystation increases the value of the chapter.

The newest dicoveries in the field of coccidiology have been made by electron microscopy. The information emerging from such studies is summarized by E. SCHOLTYSECK in the fourth chapter, on more than 40 pages. The extraordinarily clear and concise descriptions are matched by a rich illustration material, including electron micrographs and excellent drawings. The systematic relationship of *Eimeria, Toxoplasma, Isospora, Frenkeliae, Besnoitia* and *Sarcosporidia* has been confirmed by certain ultrastructural characteristics, above all by structural details of sporozoites and merozoites. Several electron micrographs are unique pieces, doing credit to the well-organized, important pioneer work conducted by SCHOLTYSECK and his group.

The cytochemical, physiological and biochemical aspects of coccidium research are outlined by J. F. RYLEY in the fifth chapter, on about 35 pages and the sixth chapter, contributed by J. F. DORAN, deals on 70 pages with the *in vitro* cultivation of coccidia. The reader finds in these chapters a summary of the newest research results and a rich list of references to assist further investigations. In the seventh chapter P. L. LONG describes, on about 40 pages, the pathogenicity of coccidia and the pathology of the diseases caused by them. The great practical importance of poultry coccidiosis is well-known, and the veterinarian has to be familiar with the coccidial diseases of domestic mammals (cattle, sheep, rabbit, etc.) as well. The pathomechanism of coccidioses is not yet fully understood. The information summarized in this chapter may also be helpful for the veterinary practitioner, who, nevertheless, will probably miss instructions concerning the prevention and control of coccidiosis and advice in several other practical problems. Another point of veterinary interest is the

problem of immunity to coccidia, discussed by M. Elaine ROSE in the eighth chapter on 40 pages. The authoress, very correctly, does not regard host specificity and age resistance as immunological phenomena. The excellent descriptions of the nature and specificity of acquired immunity and of antibodies formed in response to coccidial infections are again not followed by practical information which would assist animal health specialists engaged in coccidiosis control. Immunobiologists, nevertheless, will find all information they need in this very well-written chapter.

During the recent years the problem of toxoplasmas has been the main source of surprise and dispute in protozoology. This is the topic of the ninth chapter, 60 pages, contributed by J. K. FRENKEL. The description informs about the development of toxoplasmas, their now fairly certain identity with the had-been taxon *Isospora bigemina*, tissue lesions and other changes caused by toxoplasmas, and the immunobiological aspects of toxoplasmatosis. Present knowledge about the spread and mode of infection of toxoplasmas is summarized. The ninth chapter deals with the still scanty information available on the incidence and development of *Besnoitiae*, *Sarcosporidia* and *Frenkelia*, now generally regarded as coccidia. In the tenth chapter R. L. DAVIS describes the technical details of protozoological research so that this part has the value of a good laboratory textbook.

Summarizing, this excellent book provides, first of all, the theoretical basic knowledge indispensable for workers engaged in coccidium research. It is therefore comprehensible that with this principal aim in mind, the authors devoted less attention than might be expected by the veterinary reader to the immunological and pharmaceutical aspects of organized coccidiosis control.

L. PELLÉRDY

C. A. OSBORNE, D. G. LOW, D. R. FINCO: *Canine and feline urology*. 417 pages, 112 illustrations. Published by Saunder Company, Philadelphia, London, Toronto 1972; £ 9.35

Nowadays the development of veterinary science follows two main tendencies: specialization and integration. Specialization is no longer limited to the individual species, because efforts for causal therapy and prevention require an exact knowledge of the relation between organ and disease. This is especially relevant in the small animal practice.

Integration, as the synthesis of various branches of veterinary science and the interdisciplinary outlook are important prerequisites of veterinary clinical studies, *viz.*, internal medicine, surgery and obstetrics. These disciplines now join efforts in investigating the diseases of the various animal species and organs on a complementary basis.

According to the reviewer's knowledge, this is the first textbook of veterinary urology ever published. It is a matter of course rather than a chance coincidence that the first volume of its kind deals with the urological problems of the dog and cat. It is not only that these species frequently suffer from urinary tract disorders, but also that diagnostic differentiation from the symptoms of other organic diseases (reproductive, nerve) is often difficult. The majority of the practitioners are able to identify the syndrome, but usually more than that is required for an effective therapy.

The volume is divided into four chapters. The clinical description of the urinary tract diseases is followed by the discussion of conditions affecting the upper and lower parts of the urinary tract.

The individual chapters are either introduced or complemented by relevant information from the fields of anatomy, physiology and laboratory diagnostics. The techniques of X-ray examination, biopsy and cystoscopy are also described. The infectious, parasitic and neoplastic diseases are discussed in the chapters dealing with the respective urinary tract part.

The volume is a valuable textbook for the small animal practitioner working at a high level. The structure of the chapters is shaped to the course of the everyday clinical routine and the book is therefore easy to consult when problems of diagnosis or therapy arise. It was obviously written with the aim to give full information on present knowledge of the field and the authors practically succeeded in fulfilling this objective. The reviewer wonders whether the prostatic gland, as a complementary genitourinary organ has been forgotten or deliberately omitted, but this circumstance does not, of course, diminish the value of the book. It is recommended to all veterinarians with special interest in the details of dog and cat urology and also the workers engaged in comparative pathology will find in it many important informations.

The typographic presentation of the volume is excellent and the technique of picture reproduction deserves special mention. The contents and presentation compensate for the high price.

Z. HORVÁTH

Walter BAIER und Franz SCHAETZ: *Tierärztliche Geburtskunde (Veterinary Obstetrics)*. Stuttgart, 1972; Ferdinand Enke Verlag. 334 pages, 90 pictures. Price DM 49.—

The demand for veterinary intervention in obstetrics and reproduction biology is increasing day by day with the progress of modern animal husbandry. Professors BAIER and SCHAETZ, internationally reputed experts of the field, have satisfied this demand by writing a book on veterinary obstetrics for the use of practitioners. The volume is essentially the successor of the textbook entitled "Tierärztliche Geburtskunde und Gynäkologie" (Veterinary Obstetrics and Gynaecology), published in 1928 and 1944, followed by a third, completely revised, edition under the title "Tierärztliche Geburtskunde" in 1958, and the present volume in 1972. This has been welcomed by all professionals with special interest in the topic. As suggested by the title, the text follows up the three main phases of the life of the female animal, *viz.*, the periods of pregnancy, parturition and puerperium, with due consideration to the related biological (physiological and pathological) events and completed with descriptions of diseases of newborn. The four main parts of the book, Pregnancy (pages 15—80), Parturition (pages 89—222), Puerperium (pages 227—291) and Newborn (pages 295—315). The first part deals in great detail with the physiology of gestation, foetal development and physiological changes of the maternal body as well as sterility and mastitis. The part describing the pathology of gestation discusses developmental disturbances of foetal envelopes and foetus and pathological alterations of the maternal organism during pregnancy. Under the heading of Parturition are discussed the physiology of parturition, the various modes of veterinary obstetric assistance and the problems of pathological parturition.

The chapters on Puerperium are the most valuable. This critical period is decisive in respect of the further fate, *viz.*, reproduction ability of the animal. More than 60 pages — almost one fifth of the volume — are devoted to the clear and concise presentation of this topic, with conclusions well founded on important clinical observations. The detailed descriptions of the pathogenesis of puerperal fever and septicaemia, and of post-partum metabolic diseases with instructions for therapy and prevention deserve special mention.

The numerous illustrations, including good pictures and artistic drawing, greatly increase the value of the book. The text is clear and concise throughout and the outstanding typographic presentation is a great merit of the publisher. Undergraduates and practitioners can equally profit from this volume.

J. HARASZTI

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РЕЗЮМЕ

## ИНФЕКЦИОННОЕ ВОСПАЛИТЕЛЬНОЕ ЗАБОЛЕВАНИЕ КЛОАКИ И ПЕНИСА У ГУСЯ

### I. НАБЛЮДЕНИЯ ПО ЭПИЗООТОЛОГИИ, КЛИНИКЕ, ПАТОЛОГИИ И ЛЕЧЕНИЮ ЗАБОЛЕВАНИЯ

И. СЕП, М. ПАТАКИ и ДЬ. НАДЬ

Описывается раньше неизвестное заболевание половых органов гусака, вызываемое тяжелые потери в крупных гусиных поголовьях. Заболевание характеризуется тяжелым поражением органов совокупления гусака с необыкновенно высоким соотношением стерильных яиц. Первым клиническим признаком является угнетенность животных, сопровождающаяся воспалением клоаки, у гусаков же покраснением и воспалением пениса и клоаки. В тяжелых случаях пролапсус пениса сопровождается его частичным или полным некрозом и отбрасыванием. В неблагополучных поголовьях в течение нескольких недель даже 10% больных гусаков может погибнуть. Микроба, напоминающая **Neisseria gonorrheae** была изолирована из изменений органов и туш погибших животных. Дается полное описание симптомов и изменений при заболевании, дальше, рекомендуемые терапевтические и профилактические мероприятия.

## ИНФЕКЦИОННОЕ ВОСПАЛИТЕЛЬНОЕ ЗАБОЛЕВАНИЕ КЛОАКИ И ПЕНИСА У ГУСЯ

### II. СВОЙСТВА ЭТИОЛОГИЧЕСКОГО ФАКТОРА И ЭКСПЕРИМЕНТЫ ПО ЗАРАЖЕНИЮ

М. ПАТАКИ

Из раньше ненаблюдающегося заболевания совокупительного органа в качестве этиологического фактора изолирован вид бактерий рода **Neisseria**. При болезни поражается клоака, а у гусаков пенис. Эпизоотология, способ заражения, гистопатологические изменения соответствовали таковым при гонорее, но в биохимических пробах изоляты вели себя по-другому чем **Neisseria gonorrheae** и все другие виды (**N. sicca, N. intracellularis, N. flava, N. subflava, N. perflava**) данного рода. Расшифровка значения этого нового вида рода **Neisseria**, который кажется специфически патогенным для гуся, требует дальнейших экспериментов.

## АППАРАТ ЭРЕКЦИИ СОВОКУПИТЕЛЬНОГО ОРГАНА ГУСЯКА И СЕЛЕЗНЯ

Э. ГУЖАЛ

Структуры, содействующие в эрекции совокупительного органа самцов птиц — это васкулярные тела, система лимфатических синусов, мускулатура клоаки и пещеристая ткань пениса. Парные васкулярные тела располагаются на вентральной поверхности клоаки и обширный лимфатический синус, окружающий клоаку, коммуницирует с пенисом при посредстве лимфатических каналов. Васкулярные тела снабжаются кровью

внутренней срамной артерией, которая, разветвляясь, образует в них густую сеть капиляров. Капиляры и лимфатические синусы выстланы непрерывными эндотелием. На сексуальный стимул мускулатура артерий и докапилярных артериол расслабляется, просветы расширяются и кровь внезапно внедряется в капиляры, вызывая этим их расширение и наступает разливание лимфы из сосудов как результат повышения гидростатического давления. Лимфа, попавшая в лимфатический синус, фильтрационным давлением и сфинктером клоаки, окружающим лимфатический синус, прокачивается дальше в пенис. Обратный процесс проходит при расслаблении. Пенис втягивается в покоящее положение эластическими волокнами, тугими при эрекции, называемыми m. rectractor penis; они содействуют однако только в ретракции вентральной части клоаки и основания пениса. Лимфа из пениса поступает в лимфатические синусы, откуда она выходит при помощи лимфатических сосудов.

## РАЗВИТИЕ КИШЕЧНОЙ ФЛОРЫ У ПОДСОСНЫХ ПОРОСЯТ

ДЬ. ШИНКОВИЧ и Б. ЮХАС

На основании бактериологического изучения желудочно-кишечного тракта, по определенной программе убитых животных, показано развитие флоры пищеварительного тракта подсосных поросят до 12-дневного возраста. Согласно полученным данным характерная для здоровых поросят флора формируется к 1,5—2-дневному возрасту и с этого времени по 12-дневный возраст существенно не изменяется. В кишечной флоре подсосных поросят сначала превалируют везде обнаруживающиеся стрептококки, превышая количеством стрептококки Д. Вегетативные и споровые формы клостридий от рода по 12-дневный возраст обнаруживаются во всех участках кишечника, но в тонком отделе отмечается уменьшение количества обеих форм.

Отдельные этапы формирования кишечной флоры указывают на наличие механизма, регулирующего биоценоз просвета, который до определенной степени является независимым от количества снаружи попадающих в кишечник бактерий.

## ЭЛЕКТРОННО-МИКРОСКОПИЧЕСКИЕ ИССЛЕДОВАНИЯ НА СТАДИЯХ РАЗВИТИЯ EIMERIA MAXIMA

### IV. ТОНКАЯ СТРУКТУРА ШИЗОНТОВ И МЕРОЗОИТОВ

Х. МЕЛХОРН

Электронно-микроскопическим методом изучается шизогония куриной кокцидии **Eimeria maxima** и сравнивается с соответствующими отправлениями других споровиков. Оказывается, что описывающиеся в работе основные отправления цикла развития вопреки имеющимся модификациям происходят от единой исходной формы, которая в настоящее время отражается в однодиогении некоторых видов.

Структура зрелых мерозитов E. maxima в принципе не отличается от таковой других аймерид. Следует однако подчеркнуть, что коноид может быть сдвиженным настолько вперед, благодаря чему он сидит на полярном кольце, чем сильно подчеркивается его роль, как органа внедрения. Подобно сказанному обнаружена очень тесная структуральная взаимосвязь между коноидом, роптриями (парными органеллами) и микронемами, на основании чего предложенное Левайн (1970) наименование «Apicomplexa» для «бывших» споровиков кажется весьма выразительным.

## БИОЛОГИЧЕСКИ АКТИВНЫЕ ОСТАТКИ В МЯСЕ И МЯСНЫХ ПРОДУКТАХ В ВЕНГРИИ В 1972 ГОДУ

Я. ТАКАЧ, З. ШИМОНФФИ и Х. Е. ЯНЧО

Образцы мяса и мясных продуктов изучались на наличие биологически активных остатков. Объектом исследований явились свиньи и мясной скот крупных хозяйств. В анализ брались образцы мяса, жирной ткани, почек и печени, дальше, таковые пастеризованной консервной ветчины, салами и сырой копченой колбасы.

Соединения хлорированных углеводородов, фосфорорганических инсектицидов, мышьяка, меди, цинка и цинк-бацитрацина изучены в 725 образцах. Результаты исследований собраны в 3-х таблицах.

Концентрация ДДТ и Линдана в жирных тканях была в 10 и 5 раз выше, чем в мясе, но она была намного ниже терпимого уровня международных предписаний.

Фосфорорганические инсектициды не обнаружены ни в образцах мяса, ни в таковых мясных продуктов.

Уровень тяжелых металлов и радиоактивных элементов, в том числе и цинк-бацитрацина был низко под допустимым уровнем международных предписаний.

Концентрация ДДТ изменилась только незначительно, таким образом с его наличием нужно рассчитывать еще долгое время.

## ИЗУЧЕНИЕ ЦИТОТОКСИЧЕСКОГО ЭФФЕКТА ТОКСИНА $Ф_2$ (ЗЕАРАЛЕНОНА) В РАЗНЫХ ОДНОСЛОЙНЫХ КЛЕТОЧНЫХ КУЛЬТУРАХ

А. ВАНИ и Э. РОМВАРИНЭ-СЕЙЛЕР

Авторами изучался цитопатогенный эффект токсина $Ф_2$ (зеараленона) в разных однослойных клеточных культурах. Обнаружено, что ткань семенников тех животных чувствительна к токсину, у которых он тормозит спермиогенез. Ткань семенников резистентных к токсину животных и in vitro не повреждалась им.

Другие виды тканей in vitro не показывали чувствительности к токсину. Этот метод авторы считают удобным для быстрого определения чувствительности разных видов животных и их тканей к токсину $Ф_2$.

## НАИБОЛЕЕ НАДЕЖНЫЙ МЕТОД ВЫЯВЛЕНИЯ ПРЕДСТАВИТЕЛЕЙ ГРУППЫ ЭНТЕРОКОККОВ

Я. ТАКАЧ и Ш. КОВАЧ

В микробиологии пищевых продуктов выявление бактерий из группы энтерококков считают доказательством загрязнения микрофлорой из фекалий. Виды данной группы — главным образом **Streptococcus faecalis** и зго варианты — в литературе неоднократно фигурировали как причина порчи пищевых продуктов и энтеральных заболеваний.

Виды стрептококковой группы сильно отличаются друг от друга в резистентности, в способности роста, в биохимической активности, в избирательности к питательным средам. Чтобы найти наиболее надежный способ выявления представителей энтерококковой группы, проведено сравнительное изучение 6 жидких и 10 плотных питательных сред.

Все представители энтерококковой группы наиболее дружно росли на натриево-азидо-декстрозной жидкой среде, содержащей 0.003% бромтимолсини и на плотной агарной среде Паккера при культивации при 35—37°С в течение 24—48 часов. Результаты исследований собраны в таблице.

## СЕРОЛОГИЧЕСКОЕ ИЗУЧЕНИЕ КОЛИБАЦИЛЛЕЗНЫХ ШТАММОВ, ИЗОЛИРОВАННЫХ ИЗ ТЕЛЯТ

Я. ВАРГА и А. Ф. ФАРИД

У 288 колибациллезных штаммов, изолированных из туш, павших от коллибациллезной дизентерии телят 91 крупной фермы, изучены биохимические свойства и антигены О и К. Для определения антигенов О применили 57 колибациллезных антисывороток О и ОК; для определения антигенов К — за исключением трех — все (1—94) колибациллезные антисыворотки К.

На основании биохимических исследований 263 изолята идентифицированы как **E. coli**, 15 — как **Klebsiella**., 5 — **Proteus vulgaris**, 3 — как **P. mirabilis**; два штамма не удалось включить в перечисленные роды. Большинство штаммов E. coli показывало био-

химические признаки, характерные для рода, но некоторые штаммы в отдельных реакциях вели себя по-другому.

150 штаммов среди всех (57%) на основании набора антисывороток удалось включить в группы О. Большая часть штаммов принадлежала к серогруппам О101, О20, О141, О8 и О7, тогда как группы О21, О23, О153, О9 и О78 отмечались редко.

За исключением одного все серологически идентифицированные штаммы содержали антиген К. Почти половина штаммов содержала антиген К(А), во формах О101 : К28(А), О101 : К30(А) и О8 : К28(А); однако обнаружены и термостабильные, ближе неидентифицированные, вероятно антигены К(А) в штаммах, принадлежащих к серогруппам О9 и О41. Другая часть штаммов содержала антигены К(В), или К(L), как правило в форме О8 : К71 (В), О153 : К71(В), О78 : К80(В) и О117 : К15(L).

## ЗНАЧЕНИЕ ПАТОЛОГОАНАТОМИЧЕСКИХ ИЗМЕНЕНИЙ ГИПОТАЛАМА У ЖИВОТНЫХ, ЗАРАЖЕННЫХ В МОЗГ ШТАММОМ ВИРУСА БЕШЕНСТВА ХЁДЕШ ФИКС

Д. КАРАССОН

Изучались патологоанатомические изменения гипоталама экспериментальных животных (обезьян резус, овец, белых мышей, сирийских хомяков и кроликов), зараженных в мозг штаммом Хёдеш фикс вируса бешенства. Автором обнаружено, что в гипоталаме экспериментально зараженных вирусом фикс животных, которые пали и были убиты на разных стадиях болезни, всегда имеются явления острого энкефалита. Глиозные и мезенхимальные инфильтрации, дальше, дегенеративные изменения нейронов здесь, как правило, были более тяжелыми, чем на других местах центральной нервной системы.

В начале инфекции более тяжелые изменения имелись в передних, а к концу, в задних частях гипоталама.

Согласно автору нейропатологические изменения в гипоталаме являются причиной хорошо известных симптомов со стороны вегетативной нервной системы, сопровождающих бешенство. Характерная последовательность симптомов объясняется последовательностью заболевания ядер гипоталама.

Данные исследований — кроме доказательства избирательной заболеваемости гипоталама в процессе патогенеза бешенства — представляют собой новый метод в экспериментальной нейрологии, поскольку применялся строго нейротропный вирус фикс.

## МАССОВОЕ ОБНАРУЖИВАНИЕ ЗАВОРОТА ВЕК СРЕДИ ЯГНЯТ

Й. ЗАЙЕР, Т. КЕГЛ и К. ШОМОДЬВАРИ

Заворот нижнего века среди метисных ягнят от венгерских мериносовых овцематок и баранов асканийский, грозненский, кавказский и прекосный меринос с 1967. года наблюдается в 3—10%-ах случаев. Заболевание глаза появлялось обычно в 10—20-дневном возрасте и примерно 60—70% животных погибло от болезни.

Наблюдения по этиологии и эксперименты по лечению болезни велись в 12 неблагополучных поголовьях. Врожденный заворот никогда не обнаруживался и из больных глаз не удалось изолировать специфическое заразное начаго. Патогистологическим изучением структуральной аномалии век обнаружить не удалось. У метисных ягнят, характеризующихся заметной складчатостью кожного покрова, возникает спазмический заворот в результате механической иритации роговицы ресницами в силу заворота нижнего века в сторону глазного яблока, так как в области век кожный покров тоже образует складки. Таким образом это состояние нельзя считать наследственным; в его возникновении в качестве предрасполагающего фактора важную роль играет рыхлость структуры подкожной соединительной ткани нижнего века. Подкожным введением 0,75—1,5 мл 70%-ного этилевого спирта в подкожную соединительную ткань нижнего века является простым, легко осуществимым и эффективным способом ликвидации заворота нижнего века. Метод зарекомендовал себя в лечении многих тысяч метисных ягнят.

## ДЕЙСТВИЕ АЗОТА В КОРМЕ НА ОБМЕН ЭЛЕКТРОЛИТОВ И ВОДЫ У ОВЦЫ

Б. ЮХАС, Б. СЕГЕДИ и М. КЕРЕСТЕШ

Изучалось распределение $^{42}K$ в крови, сыворотке и эритроцитах овцы после введения в рубец 0,6—0,8 г/кг мочевины при повышающейся концентрации аммиака в крови.

Определялись содержание аммиака в жидком содержимом рубце и в полной крови и гематокрит. Радиоактивный $^{42}K$ вводился внутривенно. Активность изучения крови, сыворотки и эритроцитов регистрировалась после 0, 1,5, 3, 4,5, 6 и 8 часов после введения мочевины.

У овец, получивших мочевину, активность $^{42}K$ в сыворотке понизилась более значительно, чем в контрольных животных и наблюдались симультанные показатели активности излучения в полной крови. В разрез с контрольными животными наблюдалось понижение активности $^{42}K$ и в эритроцитах.

Является очевидным, что повышение уровня аммиака в крови тормозит переход $K^+$ из межклеточных щелей в клетки и поощряет выхождение $K^+$ путем диффузии из клеток.

Обнаружено, что — благодаря тормозящей активности аммиака — имеется определенная взаимосвязь между повышением уровня аммиака в крови квоциентом распределения $K^+$. Чем выше уровень аммиака в крови, тем больше торможение перехода $K^+$ в эритроциты. На выражение корреляции между уровнем аммиака в крови и квоциентом распределения составлена регрессионная кривая.

## ИЗУЧЕНИЕ ЦИТОТОКСИЧЕСКОГО ЭФФЕКТА ФУЗАРИОТОКСИНА Т—2

Л. БОДОН и Л. ЗЁЛДАГ

Авторами изучался цитотоксический эффект продуцированных разными видами грибков рода Fusarium микотоксинов на тканевых культурах и кожной пробой у кролика. Тканевая культура оказалась очень чувствительным методом, при помощи которой удалось выявить наличие уже 0,005 мцг токсина Т—2. Цитологические изменения изучались на препаратах, окрашенных акридин-оранж и гематоксилин-эозином. Характерными изменениями в тканевой культуре явились решетчатая структура клеточного слоя и пикноз содержимого клеточных ядер и распад последних (кариорексис). Метод удобен и для количественного титрирования чистого токсина. Кожной пробой на кролике удавалось выявить наличие токсина до предела 0,5 мцг.

## ИНФЕКЦИИ ИНФЛЮЭНЦОЙ СРЕДИ ЛОШАДЕЙ ВЕНГРИИ, ВЫЗВАННЫЕ ПОДТИПАМИ ЛОШАДИНОЙ А—1 И ГОН КОНГ

Й. РОМВАРИ, Я. ТАНИ, ЖУЖА МАТЭ и Э. ФАРКАШ

Штаммы инфлюэнцы подтипа лошадиный А—1 были изолированы из носовых источений больных лошадей, показывающих мягкие признаки острого заболевания дыхательных путей. В неблагополучных поголовьях среди 286 образцов крови 56,6% давали положительную реакцию в пробе торможения гемагглютинации, обычно до разбавления 1 : 16—1 : 64.

Остаточные антитела к вирусу лошадиный А—2 обнаруживались только в 17,8%-ах образцов сыворотки лошадей возраста выше 5—6 лет, при тяжелой вспышке эпизоотии лошадиный А—2 в 1968. году.

Среди 188 образцов крови 39 (20,6%) дало положительную реакцию к вирусу Гон Конг спустя 5—8 месяцев после эпидемии, вызванной этим вирусом. Эти антитела скоро исчезли.

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