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ON ULTRASTRUCTURE AND MOTILITY OF SPIROCHETES

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(Received August 12, 1984)

Electron microscopical investigations of the agent of swine dysentery *(Treponema hyodysenteriae;* Harris et al., 1972) isolated from faeces of healthy pigs as well as from the colon of pigs with swine dysentery are described. Similar investigations were carried out on *T. innocens* (Kinyon and Harris, 1979), on a spirochete isolated from the caecum of rats and, finally, on *T. reiteri.*

The ultrastructure of the above microbes is different from that described for *T. hyodysenteriae* and *T. innocens*, and for spirochetes generally. Based on the results, the motility of spirochetes of the genera *Treponema* and *Borrelia* is explained. The arrangement of axial fibrils and, consequently, the type of movement, are useful for a clear and sufficient distinction between the genera *Treponema* and *Borrelia*, and *T. hyodysenteriae* has to get a new combination to *Borrelia hyodysenteriae.* The *T. innocens* strain Puppy and the spirochete from the caecum of the rat are also regarded as belonging to the genus *Borrelia.*

Keywords. Ultrastructure, motility, *Treponema hyodysenteriae*, *T. innocens*, *T. reiteri*, *Borrelia*, taxonomy.

Since the investigations of Harris et al. (1972) it has been shown that a spirochete, generally designated as *Treponema hyodysenteriae*, is the major definite aetiologic factor in swine dysentery. Only Janetschke and Kielstein (1976) proposed the name *Borrelia hyodysenteriae* for the same organism because it has wide waves and stains with aniline dyes. The criteria for the distinction between the genera *Treponema* and *Borrelia* are not sufficient for a clear integration of an unknown spirochete into one of the two genera. Apart from that, we are of the opinion that the hitherto existing description of the arrangement of axial fibrils can explain neither the shape and the motility of spirochetes nor the various types of movement of spirochetes (snake-like or corkscrew-like).

In the present paper our findings about the ultrastructure of *T. hyodysenteriae*, *T. innocens* and a spirochete from the caecum of the rat, our conclusion about the importance of ultrastructure for the genus determination of *Treponema* and *Borrelia* as well as an explanation of the motility mechanism of spirochetes are reported.

Materials and methods

Culture technique and media. The isolation of spirochetes from the colon of pigs and the caecum of rats as well as the production of pure cultures were performed with Trypticase soy agar and Trypticase soy broth (Difco) as described elsewhere (Blaha and Günther, 1982).

Bacterial strains. The following strains were investigated: 7a/81, 10/82, 12a/82 = large spirochetes from pigs with acute dysentery; 2/81 and 6/81 = large spirochetes from healthy pigs; 11/82 = large spirochetes from an adult white rat (*Rattus norvegicus*, albino, strain Wistar, female, 200 g, from the VEB Versuchstierproduktion Schönwalde); *T. innocens*, strain Puppy, from the Czechoslovak Collection of Microorganisms, deposited there under the number CCM 6161, and *T. reiteri* (obtained from Dr. István Horváth, Budapest).

Electron microscopy. Scanning electron microscopy with critical point drying: The spirochetes were concentrated by centrifugation of fermenter cultures at 2000 × *g*, dehydrated in alcohol and transferred to distilled amylacetate on filter paper. Critical point drying was carried out with CO_2 in the Critical Point Dryer (Fa. Balzers Union). The preparations were coated with gold (ca. 50 nm) in the Sputter device (Fa. Balzers Union) and examined with the scanning electron microscope Tesla BS 300. Transmission electron microscopy: Negative staining: Unfixed spirochete suspensions were dropped on a glass slide. Zaponlack-coal-coated grids were put on the drops. The grids taken off were begun to dry and stained in 2% phosphotungstic acid (pH 7.2).

Cross sections: Spirochetes sedimented by centrifugation at 2000 × *g* were fixed with glutaraldehyde and osmium tetroxide, dehydrated in acetone with simultaneous staining with phosphotungstic acid and uranyl acetate after Wohlfahrt-Bottermann, and embedded in Micropal or Durcupan.

The preparations were examined in the electron microscope SEM 3-2 (VEB Werk für Fernsehelektronik, Berlin).

Results

Phase-contrast microscopy of formalin-fixed pure cultures of swine dysentery spirochetes (Fig. 1) shows the same typical picture as the living forms in wet preparations. Latter clearly reveal a fast, snake-like movement (sine-curve-like flexion of the cell body). Scanning electron microscopy after critical-point drying allows a three-dimensional presentation of the wound microorganism (Fig. 2). Contrary to all expectations, spirochetes were found wound both to the right and to the left within one preparation (i.e. within one strain). Figs 3, 4, 5 and 6 show the three main structural elements of the swine dysentery agent, such as central protoplasmic cylinder, axial fibrils wound around it and

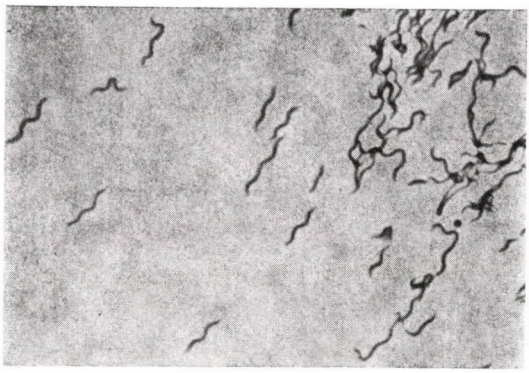

Fig. 1. Pure culture of the swine dysentery spirochete, phase contrast. ×660

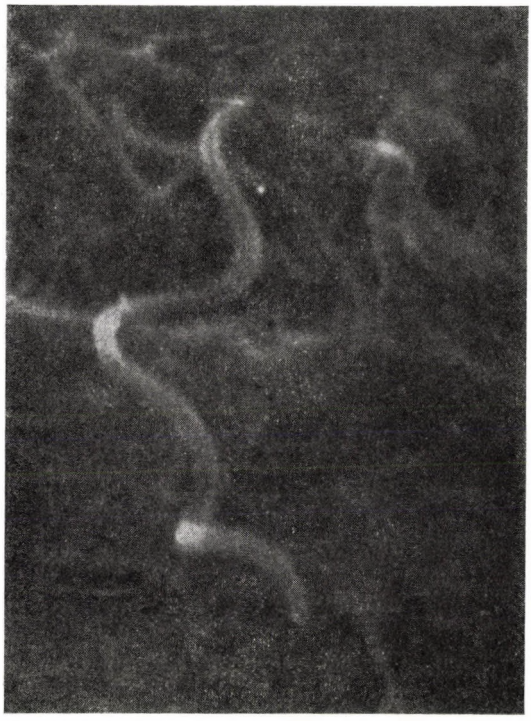

Fig. 2. Swine dysentery spirochete, SEM. ×11,000

an outer envelope surrounding both. The arrangement of the axial fibrils is shown in Fig. 3: Two fibril bundles wind oppositely round the cell body, they run from one end of the bacterium up to the other end, overcross each other several times and are subterminally fixed at both sides. The cross-points are always in the concave part of the helix. During cell division, both daughter cells

are still held together by the fibrils and the outer envelope, while the protoplasmic cylinder is already divided (Fig. 4). The cell division is finished with the tearing of fibril and envelope remnants by jerky movements ("lashing") of the daughter cells. The different arrangement of the fibrils in the cross-sections (Figs 5 and 6) is due to the specific position of the cut. In Fig. 5 the spirochete is cut directly across at the cross-point of fibrils. In Fig. 6, however, it is cut at a position at which, before or after the crossing, both bundles are placed side by

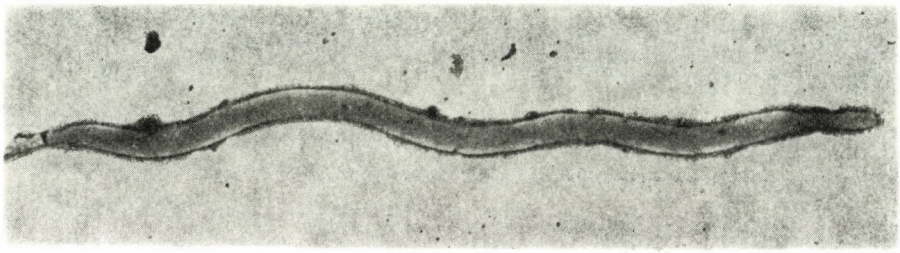

Fig. 3. Swine dysentery spirochete with five cross-points of the two axial fibril bundles, Negative staining, TEM. ×15,000

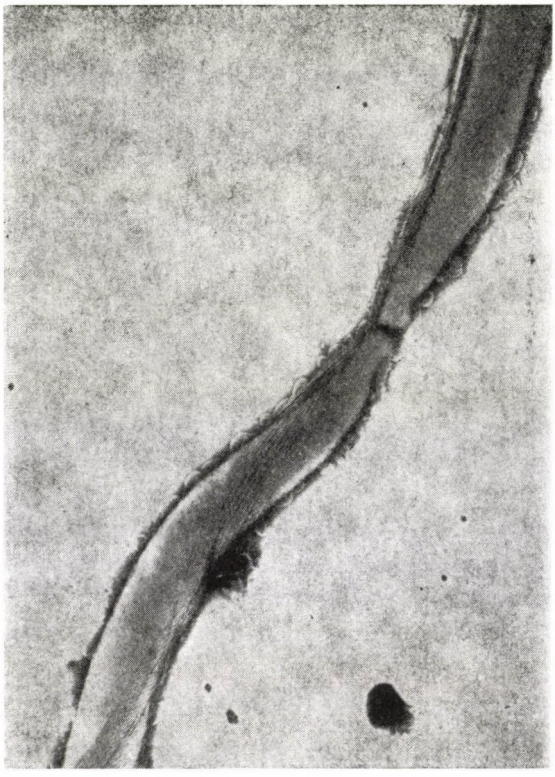

Fig. 4. Site of cell division of swine dysentery spirochete. Negative staining, TEM. ×20,000

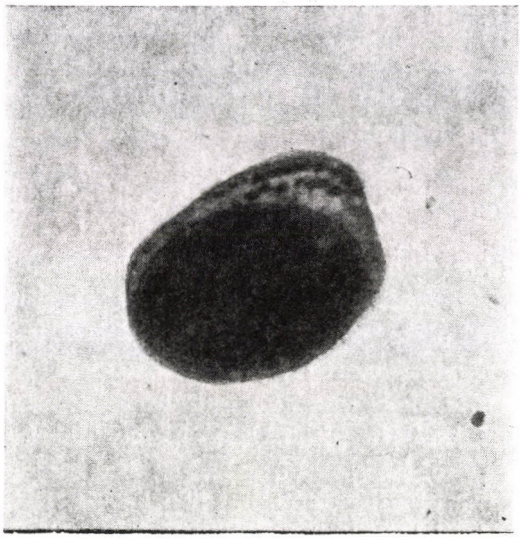

Fig. 5. Cross-section of swine dysentery spirochete cut across at a cross-point of the two bundles of axial fibrils. ×80,000

Fig. 6. Cross-section of swine dysentery spirochete cut across beside a cross-point of the two bundles of axial fibrils. ×80,000

side to separate in one direction and to overcross in the other direction. All examined strains exhibit the same arrangement of fibrils. The average bacterial dimensions and fibril numbers of the strains are summarized in Table I. Contrary to the swine dysentery spirochetes the *T. reiteri* strain shows only one bundle of axial fibrils and several tresses of cytoplasmic tubules (Fig. 7).

Fig. 7. Treponema reiteri with four axial fibrils of the one axial fibril bundle and several cytoplasmic tubules. Negative staining, TEM. ×80,000

Discussion

Smibert (1974) and Canale-Parola (1977, 1978) described the arrangement of axial fibrils for all members of the order Spirochaetales as follows: the fibrils are subterminally fixed at each end of the cell, they only run over about two-thirds of the cells and end up unfixed after having merely overlapped in the middle (Fig. 8). This structure was also described (Harris et al., 1972; Kinyon and Harris, 1979; Molnár, 1979; Molnár, 1981; Schleicher, 1977) for *T. hyodysenteriae*. We failed to confirm this structure for the swine dysentery agent (Figs 3 to 6) and we do not agree with the assumption expressed by Yoshii (1978), Yoshii et al. (1979) and Stepan and Johnson (1981) that the helical formation, i.e. left or right winding of spirochetes, is species-specific in each case. The close study of the snake-like movement of swine dysentery spirochetes in phase contrast and of the corkscrew-like movement of *T. pallidum* and *T. reiteri* in the dark field as well as the analysis of electron micrographs of spirochetes by us and by other investigators (Canale-Parola, 1977, 1978; Hovind-Hougen, 1974*a*, 1974*b*, 1974*c*; Jackson and Black, 1971; Listgarten and Socransky, 1964*a*, 1964*b*, 1965) allow us to draw the conclusion that spirochetes have not only one, but two different structural principles:

(1) All closely-wound spirochetes rotating around their imagined longitudinal axis without changing their helical formation have only one bundle of

Fig. 8. Scheme of the general structure of spirochetes according to Canale-Parola (1977)

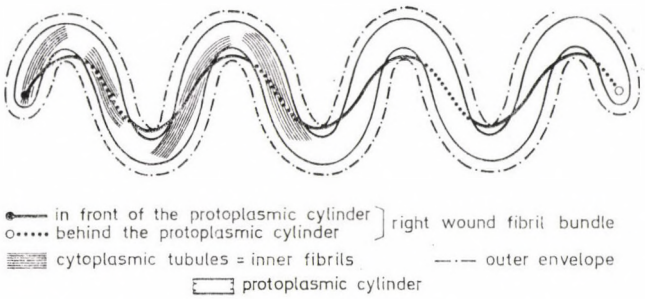

Fig. 9. Scheme of the structure of bacteria of the genus *Treponema*

few axial fibrils wound round the cell body; the bundle runs over the cell body and is inserted at both ends. Inside the protoplasmic cylinder, there are tubular structural elements which we found in a *T. reiteri* strain and which are described for treponemes as "inner fibrils" (Horváth, 1972; Jackson and Black, 1971) and finally as "cytoplasmic tubules" (Hovind-Hougen, 1974*a;* 1976). These spirochetes can only be wound either to the left or to the right (Fig. 9).

(2) All largely-wound spirochetes with snake-like movement caused by three-dimensional flexions of cell body have two fibril bundles oppositely wound round the cell body. They run over the whole cell body, overcross each other several times and are inserted at both ends (Fig. 10). Internal tubular structural elements have never been seen in this type of spirochetes. These spirochetes can be wound both to the left and to the right for they continually change their spiral direction.

These two different arrangements of axial fibrils result in two different motility mechanisms:

Movement Type I

The probably contractile internal elements of all closely-wound spirochetes cause their typical corkscrew-like movement (Horváth, 1972). The one axial fibril bundle of this type of spirochetes is responsible for their ability to make a "lashing" movement (an irregular jerky movement of the whole cell body, which is often seen before cell division).

Movement Type II

The two oppositely-wound axial fibril bundles of all largely-wound spirochetes cause their typical snake-like movement. Due to the alternating concentrations of both fibril bundles, a left-right flexion of the elastically stretched cell body in the form of three-dimensional sine-curve-like vibrations is caused, with the contracted bundle lying on the interior side of the helix and a crossing of both fibril bundles occurring after half a winding. A hydrodynamic effect is

produced by that vibration (Jahn and Landman, 1965). The microorganism is able to move forward or backward in a snake-like manner in the liquid environment.

The complicated hypothetic explanations, such as the rotation of fibrils between envelope and protoplasmic cylinder (Berg, 1976) or the lengthening of the shorter fibril bundle and the shortening of the longer one of daughter cells after division (Canale-Parola, 1978) are no longer necessary. The arrangement of fibrils described by us does not only explain the motility with the two types of movement, but also offers a simple explanation of the division process. In case of the ultrastructure detected by us only a new insertion of fibrils at the protoplasmic cylinder in front of and behind the preformed site of division is necessary. The "lashing" of daughter cells often observed in living spirochetes during the division process up to the mechanical dividing of envelope and fibril rests already presupposes an independent motility of the new cells before division. Such an immediate independent motility cannot be explained by the arrangement of fibrils assumed up to now, since, according to the scheme of Canale-Parola (1977); Fig. 8, the division of those microorganisms would produce daughter cells which considerably differ from the pattern of the mother cell (one fibril bundle would run over the whole cell body and the other would be extremely short). The criteria available for the distinction between *Treponema* and *Borrelia* do not allow a clear classification of bacteria in the two genera. As we have shown (Table I), there are spirochetes with many fibrils and those with few fibrils among spirochetes with wide waves, i.e. the number of axial fibrils is not a suitable criterion for the taxonomy of these two genera of spirochetes. The arrangement of fibrils, however, and thus the type of movement, allow a clear distinction. According to these criteria, all parasitizing anaerobic spirochetes *(Borrelia* and *Treponema)* are to be taxonomically classified as follows:

(1) All species with one bundle of axial fibrils, cytoplasmic tubules and a corkscrew-like movement belong to the genus *Treponema* (Fig. 9).

Table I

Data on the ultrastructure of examined strains

Strain	Bacterial diameter (μm)	Fibril diameter (nm)	Number of fibrils per bundle	Number of fibril bundles
2/81	0.35–0.50	20–25	8–10	2
6/81	0.60–0.70	20–25	10–12	2
7a/81	0.30–0.40	15–20	15–18	2
10/82	0.40–0.50	12–20	10–20	2
11/82	0.40–0.50	12–20	12–18	2
12a/82	0.25–0.30	12–20	4–6	2
CCM 6161	0.20–0.35	12–20	4–6	2
Treponema reiteri	0.20–0.25	15–20	4	1

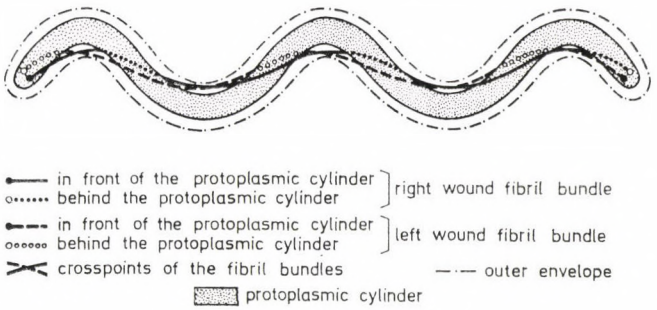

Fig. 10. Scheme of the structure of bacteria of the genus *Borrelia*

(2) All species with two oppositely wound bundles of axial fibrils and a snake-like movement belong to the genus *Borrelia* (Fig. 10).

According to the description of the type of movement of *B. anserina* and *T. pallidum*, both species retain the type species of their genera also after the above-mentioned taxonomic criteria.

The species *T. hyodysenteriae*, however, is to be renamed into *B. hyodysenteriae* comb. nov., and *T. innocens* strain Puppy and the large spirochete of the rat caecum also belong to the genus *Borrelia*.

For a new combination of further species of the genera *Borrelia* and *Treponema*, which is possibly necessary, these should be examined by electron microscopy and their movements should be analysed.

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MEAT QUALITY CHARACTERISTICS OF SOME PIG BREEDS IN HUNGARY

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A comparative analysis of meat quality results obtained for some pig breeds used in Hungary was performed on the basis of pH_1, pH_2 and Göfo values and organoleptic examination. The results indicate a 20 to 30% incidence rate of adverse meat quality in the pig breeds of largest populations in Hungary. In the Duroc breed the frequency of objectionable meat quality is 6.5%. The influence of seasonal and meteorological conditions and the site of examination on meat quality can be more expressed than the effect of breed.

Further standardization of the test methods and conditions of application is needed to allow a better comparison between breeds and between performance-testing stations.

Keywords. Pig, breed, meat quality, pH, meat colour.

So far only indirect data have been available on the meat quality of Hungarian pig breeds. The data are based on experimental measurements made at slaughterhouses, and on halothane testing of hybrids performed within the frames of surveys. Since the introduction of regular meat-quality control in fattening-performance testing it has by now become possible to get a well-grounded, although not full, picture of the meat-quality characteristics of our pig breeds.

In their investigations made on very few pigs belonging to breeds used in Hungary, Kellner et al. (1979) estimated the average incidence rate of the PSE (pale, soft, exudative) syndrome at 14, 28 and 17% in the Hungarian Large White, Swedish Landrace, and Estonian breeds, respectively.

Halothane testing of hybrid lines for stress susceptibility performed by Klosz et al. (1979), also on few pigs, revealed a 30 to 40% halothane-positivity for the Pietrain and Belgian Landrace, and a less than 5% positivity for the Large White and Dutch Landrace. The Hampshire breed was free from halothane-sensitivity. The results of halothane tests performed in different pig populations (Kovách et al., 1983) indicated the following halothane-positivity rates: less than 5% for the meat-type pig breeds and the Hampshire, 7–10% for the Swedish, Danish and English Landrace, and 40–55% for the Belgian and German Landrace.

Materials and methods

At the end of 1981, all performance-testing stations of the Hungarian Inspectorate for Animal Nutrition and Breeding introduced pH measurement in the left body-half 45 min (pH_1), and in the right body-half 24 h, after slaughtering (pH_2), together with the measurement of meat colour by a Göfo instrument 24 h after slaughtering in the m. longissimus dorsi, on a cut surface behind the 13th vertebra. In addition, the general impression obtained of the meat was evaluated subjectively: based upon the organoleptic evaluation of colour, structure and moisture content the best-quality meats received 3 points. This estimation was performed at the same sites as pH and meat colour determination, and also in the thigh muscle, 24 h after slaughtering. The time elapsed since then allows us to evaluate the applied methods and the breeds included in the study. The data obtained for 1982 were evaluated by computerized, comparative statistical procedures and regression analysis.

Results and discussion

Of the pig breeds used in Hungary, the Duroc breed gives the best-quality meat (Table I); this means a meat of the darkest colour, the lowest variability in pH and meat colour, and the lowest incidence rate (6.5%) of meats of adverse quality (meat defects). The Duroc breed is followed by the Hungarian Large White showing a 21% frequency of PSE (pale, soft, exudative) + DFD (dark, firm, dry) meat defects, and then by the Estonian pig. Of the purebred breeds, the Hungarian Landrace showed the highest frequency (about 30%) of meats of objectionable quality. Taking only the pH value as a basis of meat-quality estimation, the Hungarian Large White and Estonian breeds are qualified as PSE-carriers in about 15%, whereas the Hungarian Landrace in about 19%.

Table I

Meat properties by breed

Breed	n	pH_1	pH_2	Göfo	Organoleptic evaluation	PSE %	DFD %
Hungarian Large White	1471	5.91 ± 0.26	5.63 ± 0.26	72 ± 7.7	2.10 ± 0.76	18.2	2.8
Estonian	286	5.93 ± 0.27	5.68 ± 0.23	71 ± 8.8	1.94 ± 0.76	23.1	4.5
Hungarian Landrace	663	5.86 ± 0.32	5.59 ± 0.32	69 ± 10.9	1.90 ± 0.85	27.0	2.9
Duroc	108	5.88 ± 0.17	5.64 ± 0.14	74 ± 4.6	2.41 ± 0.61	5.6	0.9
Significant difference: $P < 0.05$		0.04	0.03	2	0.14		

Table II

Correlations (r) between meat properties
(n = 3079)

	pH_1	pH_2	Göfo	Organoleptic evaluation	Fat, %	Valuable meat, %	Ambient temperature at slaughtering, °C
pH_1		*0.73+*	0.23+	0.21+	0.02	−0.03	−0.15
pH_2			*0.31+*	0.26+	−0.06	0.04	*−0.23+*
Göfo				*0.55+*	0.07	−0.06	−0.19+
Organoleptic evaluation					0.03	−0.05	−0.02
Fat, %						−0.83+	0.02
Valuable meat %							−0.09
Ambient temperature at slaughtering, °C							

+ = $P < 0.05$.

The average incidence of DFD meats is not significant; this is easy to understand because the pigs are usually transported only to short distances and are slaughtered after a short resting period; thus, the probability of their getting exhausted is low.

The high values of standard deviation also indicate that these breeds are affected with hereditary abnormalities. To a certain extent, the position of the breeds as compared to one another can be influenced by the lack of sufficient data on the individual breeds, and also by the fact that the testing stations exert different effects on the breeds examined. In any case, it can be seen from the foregoing that in our breeds the incidence rate of meats of objectionable quality is considerable.

The interrelationships existing between the qualitative properties of the meat indicate (Table II) that the pH_2 value is in close correlation with the pH_1 value. Meat colour is slightly more correlated with the pH_2 than with the pH_1 value, therefore, it is expedient to perform the Göfo measurement 24 h after slaughtering. The results of organoleptic evaluation show a moderate correlation with meat colour and a weak correlation with meat pH, indicating that organoleptic judgement is made primarily on the basis of meat colour. When the Göfo measurement will be performed regularly, organoleptic evaluation can be omitted. The correlations existing between the qualitative and quantitative properties of the meat are usually very weak ($0.00 < r < 0.11$); there are no differences between the breeds in the magnitude and direction of the correlations.

Ambient temperature at slaughtering is in a weak negative correlation with meat quality; the higher the ambient temperature the lower is the meat pH, the lighter is the colour of the meat and the higher is the relative incidence of PSE meat.

According to the results of data processing by multivariate regression analysis, the above properties jointly do not determine the individual qualitative characters better than does the strongest component alone; thus, estimation and expression of meat quality in this way do not seem to be effective.

Factors other than the genetic characteristics of the breeds can also influence meat quality indices. When meat-quality indices were examined within a breed, it was striking that there were differences between performance-testing stations in almost all the parameters examined. This can be attributed to the diversity of measurement techniques and methods used at the different stations, to the different genetic background of the pig stocks examined at the different stations, and to the different local environmental effects prevailing at the sites of slaughtering. The analysis of these factors allows us to improve the methods and their application, and draws attention to the need to standardize these methods and to elaborate and introduce a uniform practice.

The qualitative properties of the meat are influenced by meteorological conditions as well (Table III). Differences in pre-slaughtering temperature result in as large, or even larger, differences in all parameters studied as the effect of the breed. With the decrease of temperature the pH_1 and pH_2 values significantly increase and the meat becomes darker. The results of organoleptic examination do not follow the changes occurring in the instrumentally-measured properties. Compared to the great reliability of instrumental measurements, the high (30 to 40%) standard deviation of organoleptic examination indicates that, due to subjective factors, the latter method is rather unreliable.

Table III

Influence of ambient temperature and season on meat properties

	n	pH_1	pH_2	Göfo	Organoleptic evaluation
1) *Temperature effects*					
10–20 °C	1255	5.85	5.56	69	1.97
0–10 °C	1039	5.91	5.62	70	1.97
(—10)– 0 °C	657	5.94	5.72	73	2.03
<–10 °C	128	5.98	5.77	75	1.97
Significant difference $P < 0.05$		0.05	0.04	2	0.16
2) *Seasonal effects*					
December 1981–February 1982	646	5.98	5.77	75	2.02
March–May 1982	844	5.92	5.60	70	1.86
June–August 1982	774	5.83	5.56	70	2.00
September–November 1982	595	5.86	5.56	68	2.03
Significant difference $P < 0.05$		0.03	0.03	2	0.09

The seasonal effects (temperature effects summarized for a longer period) are of the same direction as those of the changes of temperature. The seasons taken in the meteorological sense exert a significant influence on meat quality parameters. Winter has an outstanding effect: the pH_1, pH_2 and Göfo values are the highest in winter. The differences between seasons are, for the most part, significant in all directions. This allows us to conclude that, in some way or other, seasonal meteorological effects should be eliminated from the evaluation.

Standardization of methods is essential to allow a better comparability and higher reliability of the results. To achieve this, in addition to using uniform techniques and methods for pH and meat colour measurements, the greatest possible standardization of all conditions of slaughtering is needed. Under such conditions meat quality control can be exercised by standardized methods and can serve as a basis for elaborating selection criteria. The data obtained in fattening-performance testing can be utilized for breeding purposes in different ways. The example and efforts of other countries show that the qualitative properties of the meat can be considered either separately, in the form of qualitative indices, or in a complex form, together with other parameters, in selection. A satisfactory basis for such an approach can be established by evaluating the results to be obtained during a further 1 or 2 years. In any case, selection for improving meat quality on a national scale cannot be imagined without support from the meat industry.

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HAEMONCHUS CONTORTUS: EFFECT OF pH AND TEMPERATURE ON SOME DEHYDROGENASES

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Activity of lactate dehydrogenase (LDH), malic enzyme (ME), isocitrate dehydrogenase (ICDH) and malate dehydrogenase (MDH) was investigated in adults of *Haemonchus contortus* (Nematoda: Trichostrongylidae). LDH exhibits optimum activity (about 180 nmoles/min/mg protein) at a pH of 6.6 and a temperature of 37 °C; ME (about 19 nmoles/min/mg protein) at pH 7.4 and 27 °C. Similarly, the pH optimum for ICDH (about 140 nmoles/min/mg protein) and MDH (about 30 nmoles/min/mg protein) is 9.0 and 7.8, respectively. Optimum temperature is 32 °C for both enzymes. The possible role of these enzymes in the metabolism of the parasite is discussed.

Keywords. *Haemonchus contortus*, dehydrogenases, pH, temperature, metabolism.

Dehydrogenases of adult *Haemonchus contortus*, involved in carbohydrate metabolism, were investigated earlier (Kaur and Sood, 1982*a*, *b;* 1983) in the subcellular fractions. However, their activity, for example that of ICDH, is too low to account for the observed functional tricarboxylic acid cycle (Ward and Huskisson, 1978). In larvae, no ICDH and aconitase could be demonstrated in the particulate fraction (Moon and Schofield, 1968). However, a very high activity is present in the soluble fraction.

Therefore, in the present study, enzyme activity has been assayed in the whole homogenate, not in the subcellular fraction. Also, effect of pH and temperature on their activity has been investigated.

Materials and methods

Adult *H. contortus* of both sexes were recovered from the abomasa of goats *(Capra hircus)* procured from the local abattoirs. The worms were thoroughly washed in saline in order to remove the adhering material.

Worms were homogenized in 0.25 mol/l sucrose for 5 min and centrifuged for 2 min at 2000 rpm to remove the cell debris. The supernatant thus obtained was employed for the assay of various enzymes.

Lactate dehydrogenase (LDH), EC 1.1.1.27, isocitrate dehydrogenase (ICDH), EC 1.1.1.42, and malate dehydrogenase (MDH), EC 1.1.1.37 were

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estimated by the method of Bergmeyer (1974), and malic enzyme (ME), EC 1.1.1.40, by the method of Freedland (1967). Change in extinction was observed at 30-s intervals for 3 min at 340 nm. Protein content was measured by the method of Lowry et al. (1951).

For studying the effect of pH, 0.05 mol/l phosphate buffer in the case of LDH and ME, and 0.05 mol/l tris-HCl buffer in the case of ICDH and MDH, were used, in the pH range of 6.2–9.4. The pH at which the maximum activity was obtained was selected for studying the effect of temperature. The range of temperature selected was 22–52 °C.

Results

The results are given in Table I and Figs 1–3. With the increase of pH beyond 6.6, there was a continuous decline in the activity of LDH (Fig. 1). At pH 9.0, there was a slight increase, followed again by a decrease. With increase in temperature, there was an increase in the activity, optimum being attained at 37 °C. This was followed by a decrease and again an increase at 47 °C, whereafter the activity remained constant.

There was a progressive increase in the activity of ME (Fig. 2) with increase in pH, optimum being obtained at 7.4. Thereafter it declined, followed first by an increase at 8.6 and then a drastic decrease. No enzyme activity was demonstrable at pH 9.4. With temperature, no particular trend was evident, optimum activity being attained at 27 °C. There was no significant difference in the activity at 37, 47 and 52 °C.

Activity of ICDH (Fig. 1) increased with the rise of pH, with an optimum at pH 7.8. This was followed by a continuous decrease. With increase in temperature, there was a progressive increase in activity with an optimum at 32 °C. Thereafter, there was no significant change.

With increase in pH, there was a progressive increase in activity of MDH (Fig. 3). However, at pH 7.8, there was a dramatic increase, followed by a con-

Table I

pH and temperature optima of some dehydrogenases of *H. contortus*

Enzyme	Optimum pH	Optimum temperature (°C)
LDH	6.6	37
ME	7.4	27
ICDH	9.0	32
MDH	7.8	32

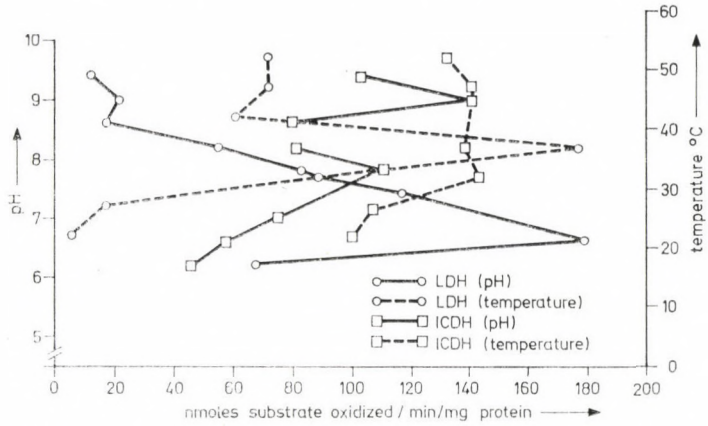

Fig. 1. Effect of pH and temperature on LDH and ICDH in *Haemonchus contortus* (Rud., 1803)

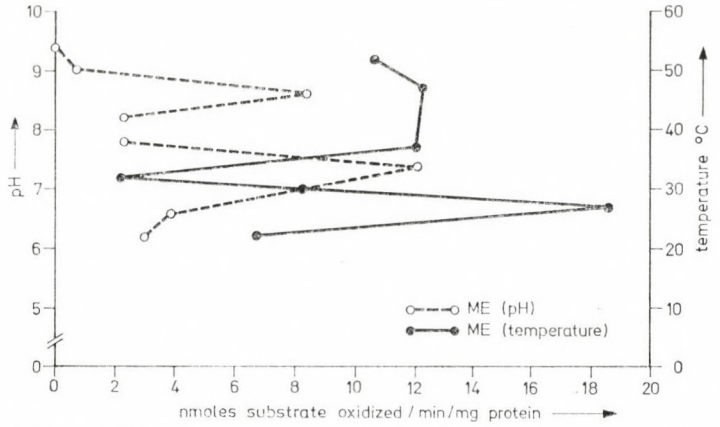

Fig. 2. Effect of pH and temperature on ME in *Haemonchus contortus* (Rud., 1803)

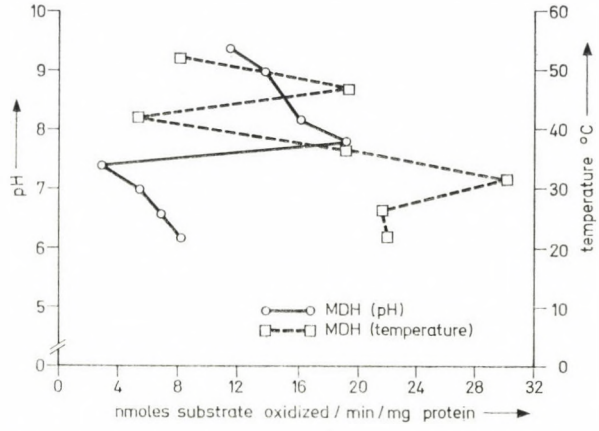

Fig. 3. Effect of pH and temperature on MDH in *Haemonchus contortus* (Rud., 1803)

tinuous decrease. With increase in temperature beyond 32 °C (optimum), there was a decrease in activity. At 37 °C and 47 °C, there was no difference in enzyme activity.

Discussion

LDH catalysing the conversion of pyruvate to lactate under anaerobiasis is present in several nematodes (Anwar et al., 1977; Walter and Schulz, 1980; Kaur and Sood, 1982*a*). In adult *H. contortus*, it exhibits an optimum activity at pH 6.6 and at a temperature of 37 °C (present studies). In the soluble fraction, a very low activity is present (Kaur and Sood, 1982*a*). Presently, a very high activity has been observed in the whole worm homogenate. High activity accounts for the anaerobic breakdown of carbohydrates as reported by Ward (1974), though it is capable of aerobic metabolism, too.

ME catalysing the conversion of malate to pyruvate is present in other nematodes, too (Anwar et al., 1977; Kaur and Sood, 1982*b*). It exhibits an optimum activity at pH 7.4 and at a temperature of 27 °C. In contrast to LDH, ME is of higher activity in the soluble fraction than in the whole homogenate. This is possibly due to its near absence in the particulate fraction.

ICDH catalyses the oxidative β-decarboxylation of isocitric acid to α-ketoglutaric acid. Most of the tissues contain two types of ICDH, of which the NAD-dependent, present only in mitochondria, is involved in the functioning of the ICA cycle. The other (NADP), present in both the mitochondria and cytoplasm, is associated with anabolic processes. In *H. contortus* the latter type has been investigated. It exhibits lower activity in the mitochondria (Kaur and Sood, 1983) than in the whole homogenate. Optimum activity is attained at pH 9.0 and 32 °C.

MDH, catalysing the conversion of malate to oxaloacetic acid, is more active in the mitochondrial fraction of adult *H. contortus* (Kaur and Sood, 1983) than in the whole homogenate. Optimum activity is attained at 32 °C and pH 7.8.

High activity of both LDH and ICDH supports the view of Ward (1974) that the adult worm utilizes glucose both aerobically and anaerobically. High activity of LDH also accounts for the 80–100% catabolism of glucose to lactate (Chopra and Premvati, 1977). The fact that in the presence of O_2 double amount of CO_2 is produced from glucose (Ward, 1974) indicates that there are two pathways of CO_2 production from glucose, an anaerobic and an aerobic one.

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EXPERIMENTAL LARVAL ASCARIDOSIS (*ASCARIS SUUM* GOETZE, 1782) IN WHITE MICE

I. HISTOCHEMICAL STUDIES ON THE LIVER

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Thirty-five white mice were infected with invasive *Ascaris suum* eggs and 5 uninfected animals were used as control. The larval invasion reached its peak in the liver on the 5th day post-infection (p.i.) as shown in squash preparations examined in a trichinoscope.

The glucosaminoglycanes (GAG) were determined with the PAS colour reaction after McManus, the DNA with the Feulgen–Rossenbeck colour reaction.

The glucosaminoglycane content was lower during the larval invasion than in the control livers, and increased in those observed between the 3rd and 9th days p.i.

The DNA content in the hepatocyte nuclei was near the control values on the 6th day and exceeded them afterwards.

Keywords. *Ascaris suum*, larval ascaridosis, liver, mouse, deoxyribonucleic acid, glucosaminoglycanes.

In the recent 20 years attention has been given to damages caused in the internal organs by migrating *Ascaris suum* larvae in infected pigs (Betts, 1954; Bindseil, 1972; Bogdanovitsh and Kanzberg, 1971; Copeman and Gaafar, 1971, 1972; Devaele et al., 1972; Eriksen et al., 1980; Feder et al., 1972; Gaur et al., 1971; Lopez, 1983; Moncol and Batte, 1967; Roneus, 1966; Roneus and Swahn, 1969; Zendulka, 1960). Considerable economic losses in pig breeding have been pointed out (Bhowmick, 1964; Boisvenue et al., 1968; Devaele et al., 1972; Fülleborn, 1921, 1922; Harsanyi, 1973; Hoeppli et al., 1949; Jenkins, 1968; Lamina, 1964; Schubert, 1970; Smirnov, 1928; Stephenson, 1978).

Experimental studies carried out on white mice infected with eggs of *Ascaris suum* confirmed the effect of larval migration on pathological changes in the internal organs (Gaur et al., 1971; Harsanyi, 1973; Jenkins, 1968; Münnich, 1959/60; Schubert, 1970; Schubert et al., 1973; Stephenson, 1978). Histochemical studies of injured internal organs in several species of animals infected by the parasite allowed researchers to interpret the possible consequences (Dhar and Singh, 1963; Kumar and Singh, 1971; Lewert and Lee, 1955; Mercado and Brand, 1954, 1960; Sauvageau and Frechette, 1980; Sawada et al., 1956; Tochtar, 1975).

The aim of this paper was to study the effect induced by migrating larvae of *Ascaris suum* upon the behaviour of glucosaminoglycane (GAG) and deoxyribonucleic acid (DNA) in the mouse liver during experimental infection.

Materials and methods

Forty female white mice of the NMRI strain were used. Their average body weight was 34 g. Five of them constituted a control group; the remaining ones were infected gastrically with a single dose of about 5000 invasive eggs of *Ascaris suum*, Goetze 1782. On each of days 3 to 9 post-infection (p.i.) five mice were killed. The peak of larval invasion was determined in impression preparations made of the whole organ (Piekut, 1975). Larval counts were made in a trichinoscope.

Liver pieces (lobus hepatis sinister medialis) were fixed in 5% neutralized formalin solution. In order to estimate GAG level on the basis of colour intensity the preparations were stained with the PAS method as described by McManus (1948). Control preparations were digested with diastase (Pearse, 1968). Feulgen–Rossenbeck's colour reaction served to assess DNA. Optimal duration of the hydrolysis with molar hydrochloric acid of 60 °C was established at 5 min (Pearse, 1968). Intensity of the colour reaction of DNA in the nuclei of liver cells was estimated visually, by comparison.

Based on the colour intensity, final conclusions were drawn as to the content of GAG and DNA as suggested by Pearse (1968).

Results

The number of *Ascaris suum* larvae in the livers and the determination of the invasion peak are presented in Table I and Fig. 1.

On the 3rd day p.i. numerous very small GAG grains were noted in the central and intermediate lobules in liver sections stained with the PAS method. In some cases PAS-positive deposits were observed in the cytoplasm of single liver cells distributed at the periphery of the lobules. In these cases the GAG content was classified as very low (Fig. 2).

On the 4th, 5th and 6th days p.i. an enrichment of cytoplasm with larger GAG grains was observed in the liver cells close to the central vein. At the same time there was a slight decrease in the intensity of the PAS reaction. Nevertheless, the total content of GAG was low (Fig. 3).

After the 7th day p.i. an increase in number of PAS-positive grains was observed in the cytoplasm of liver cells over the whole lobules; the GAG content was scored as slightly higher and high.

Table I

The effect of invasion by *A. suum* larvae on the glucosaminoglycane and deoxyribonucleic acid contents as determined histochemically in liver cells

	Intensity of histochemical reactions							
Number of days after invasion	3*	4	5	6	7	8	9	control
Average number of larvae in mouse liver	15.0	32.4	87.8	55.0	19.2	12.4	3.4	0
Glucosaminoglycanes (PAS colour reaction according to McManus)	±	+	+	+	++	+++	+++	+++
DNA (Feulgen–Rossenbeck colour reaction)	±	+	+	++	+++	++++	++++	++

Intensity of colour reaction	Conclusions on the level (content)
± very weak	very low
+ weak	low
++ slightly stronger	slightly higher
+++ strong	high
++++ very strong	very high

* In intensive studies on larval migrations, in which over 4500 mice were infected with invasive *Ascaris suum* larvae, we failed to determine with a trichinoscope the presence of larvae in liver tissue on the 1st and 2nd day p.i.

In the central part of the control lobules, close to the central vein, the cytoplasm of liver cells was poor in PAS-positive grains. On the other hand, in intermediate and peripheral regions of the lobules there were numerous large grains and deposits of GAGs, so that their level was scored as very high (Fig. 4).

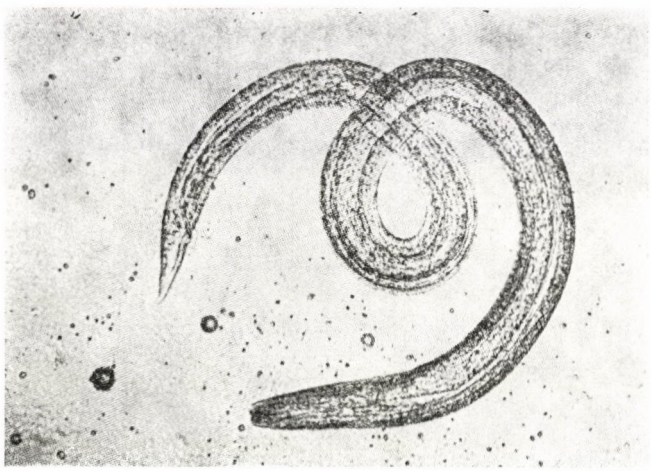

Fig. 1. Migrating larvae of *Ascaris suum* in liver of a white mouse on the 6th day p.i. ×500

The dynamics of the changes taking place in GAG levels in the liver parenchyma is presented in Table I.

The GAG level was approximately the same in the connective tissue of liver in the control and in the infected mice.

On the 3rd day p.i. most of the cell nuclei in liver sections were characterized by very weak Feulgen–Rossenbeck colour reaction, indicating a very low DNA content. Only some nuclei around the central vein contained slightly more DNA.

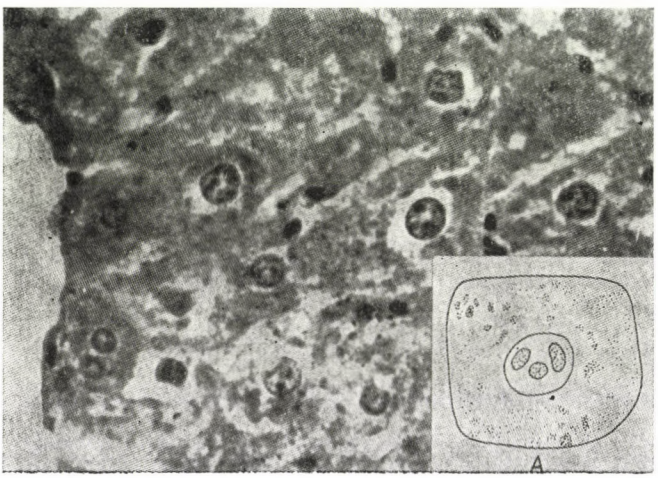

Fig. 2. Central part of liver lobule of a mouse on the 3rd day p.i.: very weak colour reaction. PAS staining according to McManus, ×920

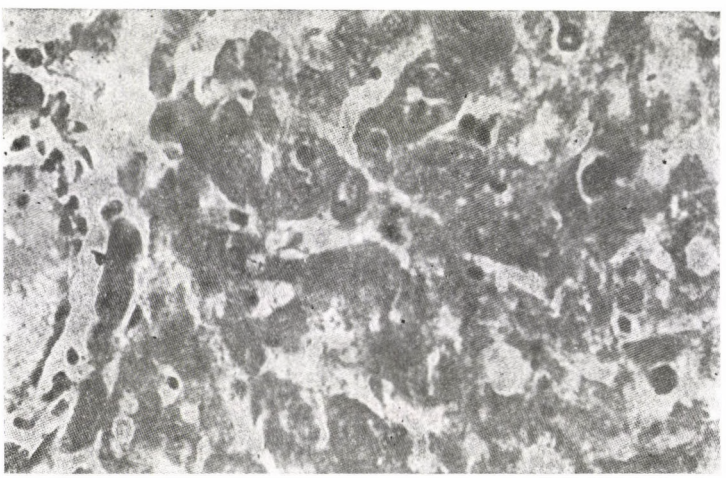

Fig. 3. Mouse liver on the 5th day p.i.: weak PAS colour reaction. PAS staining according to McManus, ×600

Fig. 4. Liver of control mouse: very strong PAS colour reaction. PAS staining according to McManus, ×750

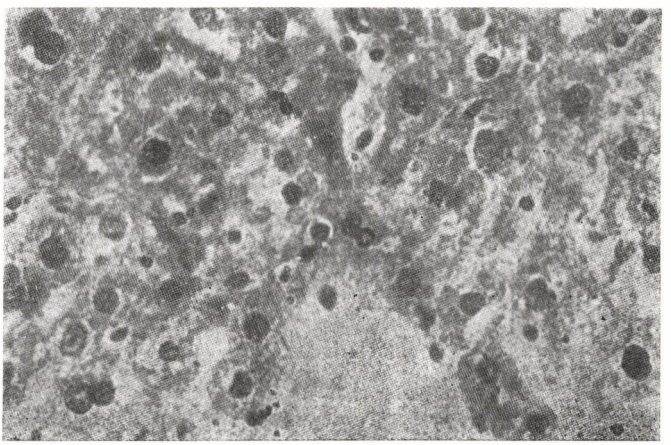

Fig. 5. Mouse liver on the 8th day p.i.: very strong DNA colour reaction. Staining according to Feulgen–Rossenbeck, ×740

On the 4th, 5th and 6th days p.i. the number of liver cells with very weak and strong DNA colour reaction increased gradually, so that DNA levels were classified as low and slightly higher. On the 7th, 8th and 9th days p.i. most of the liver cells contained nuclei with high and very high DNA levels (Fig. 5).

At the same time a strong DNA reaction was observed in the nuclei of stellate cells and infiltration cells in all liver sections of the infected mice. Moreover, an uneven diffusion of DNA was observed in the nuclei of parenchymatous liver cells, independently of the day p.i.

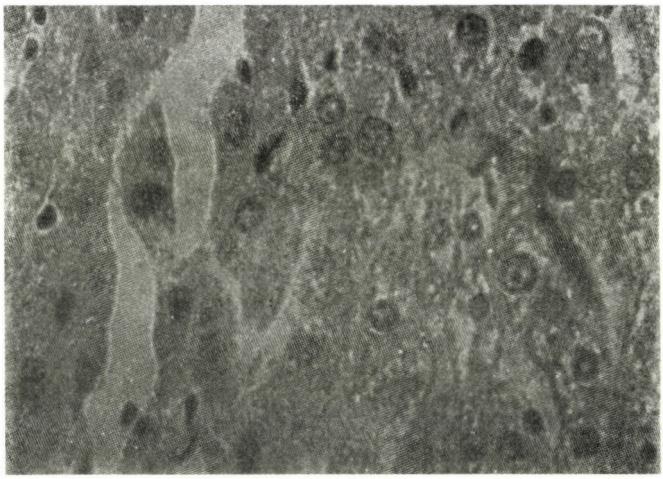

Fig. 6. Liver of control mouse: weak DNA colour reaction. Staining according to Feulgen–Rossenbeck, ×980

In the control animals DNA was evenly distributed in the nuclei of liver cells in all lobules; its level was scored as low (Fig. 6).

The behaviour of histochemical reaction in the period p.i. in the control mice is presented in Table I.

Discussion

Our experiment suggests that from the 3rd day p.i. until the peak of the invasion by migrating *Ascaris suum* larvae in mouse liver (i.e. until the 6th day p.i.), the GAG level decreases. The DNA content tends to increase already a day before. The GAG content increased on the successive days after the invasion peak, but it remained lower than in the control animals. Contrarily, DNA level increased as compared to mice free of parasite larvae and was even higher than in the latter animals.

These results are similar to those obtained by Münnich (1959/60) and Kumar and Singh (1971), who pointed to an imbalance of the compounds under study during experimental ascaridosis. However, our conclusions clearly indicate a connection between the intensity of the migrating larvae and the level of DNA and GAG.

The decrease in the GAG level in the mouse liver infected with *Ascaris suum* larvae could have resulted initially from injuries of the liver parenchyma, and subsequently from regeneration of liver cells. Similar observations were reported by other authors studying the effect of various parasites on liver (Dhar and Singh, 1963; Kumar and Singh, 1971; Lewert and Lee, 1955; Mercado and Brand, 1954; 1960; Mukanov, 1975; Münnich, 1959/60). Only Sawada et al.

(1956) found that an experimental infection of mouse liver with *Schistosoma japonicum* resulted in an increase of glycogen level in the liver cells.

GAGs constitute a source for the formation of nucleic acids, and their decrease may reflect an enhanced DNA synthesis (Malamud and Baserga, 1968; Tochtar, 1975; Yokoyama et al., 1953).

Finally, it should be taken into account that frequent occurrence of pathologic changes in the liver at the peak of an invasion by migrating larvae is caused not only by the presence of the parasite, but also by the final products of parasite metabolism (Eriksen et al., 1980; Hoeppli et al., 1949; Lopez, 1983; Münnich, 1959/60; Stephenson, 1978; Yokoyama et al., 1953).

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CHANGES IN VITAMIN E AND LIPID-PEROXIDE STATUS IN THE LAYING HEN DURING EGG SHELL FORMATION

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Vitamin E and malondialdehyde contents, and catalase and glutathione-peroxidase activities of the blood plasma, red blood cells (RBCs) and liver tissue were studied in laying hens of high egg production during the period of egg formation. Birds with inactive ovaries were used as control. The observed changes were in correlation with the alterations of sexual steroid, primarily 17-β-oestradiol, concentrations measured in that period. Hormonal changes that occurred during egg formation markedly increased the vitamin E level of the blood plasma and enhanced the lipid peroxidation processes of RBCs and liver tissue. Such changes were not found in the blood plasma, a fact suggesting that the enzyme systems of both the liver tissue and the RBCs were able to eliminate free-radical formation induced by hormonal effects.

Keywords. Vitamin E, lipid peroxidation, egg shell formation, laying hen.

Several review papers have discussed the functional anatomical and endocrinological aspects of reproductive-biological processes of birds (Bell and Freeman, 1971; Lofts and Murdon, 1973). The anatomical and endocrinological questions of egg formation have also been reviewed (Gilbert and Pearson, 1982). The above-mentioned authors gave a consistent description of the hypothalamus–hypophysis axis regulating the ovulation processes, emphasizing the importance of the luteinizing hormone (LH) not only in ovulation but also in egg formation processes (Sharp, 1980).

Besides the hypothalamus–hypophysis axis, the importance of sexual steroids should neither be neglected. Earlier studies have confirmed the fact that, although birds do not have a corpus luteum (CL), their preovulatory follicles do secrete progesterone. Besides progesterone, the synthesis of compounds necessary for the formation of the egg yolk material is regulated by oestrogens, primarily 17-β-oestradiol, in the liver (Gilbert and Pearson, 1982). In the bursting of follicles and passage of the egg through the reproductive tract, prostaglandins have an important role (Hertelendy, 1980). The synthesis of egg yolk occurs in the first phase of egg formation when, in addition to the incorporation of essential proteins, lipids and carbohydrates, that of the vitamins indispensable for embryonic life takes place (Gilbert and Pearson, 1982). These highly material- and energy-intensive changes are enacted in a very short

time and, presumably, constitute an overload for the vitamin E and lipid-peroxide metabolism of the organism.

The lipids needed for the build-up of egg yolk material mostly consist of polyunsaturated fatty acids which show increased sensitivity to damage caused by peroxidation. The purpose of the present study was to assess changes occurring in the vitamin E and lipid-peroxide status of high-producing laying hens during the period of egg formation. An attempt was made to bring these changes into connection with the hormonal changes (alterations of sexual steroid levels) that take place during egg shell formation.

Vitamin E possesses an antioxidant activity. Besides vitamin E, among other enzymes, catalase and glutathione-peroxidase, monitored by us in the present study, also take part in the elimination of free oxygen radicals formed in the course of metabolic processes.

The primary substrate of catalase is hydrogen peroxide (Jones, 1982), while for glutathione-peroxidase, in addition to hydrogen peroxide, lipid-peroxides and lipid-hydroperoxides serve as substrates. The effects of glutathione-peroxidase include the reduction of cyclic endoperoxides formed during prostaglandin biosynthesis (Flohé et al., 1971).

If iron is present in the system, the end-product of lipid peroxidation will be malondialdehyde (De Duve and Hayaishi, 1978), which, because of the not absolutely specific measurement method, was determined as a thiobarbituric acid- (TBA-) reactive product.

Materials and methods

Fifty-two weeks old "TETRA SL" laying hens (Agricultural Combinate, Bábolna, Hungary) were used. The birds were at the peak of egg production. According to the stage of egg-shell formation, they were allotted to the following groups (the status of reproductive organs and the location of the egg was examined by palpation through the cloaca):

1. Inactive: Birds with inactive (atrophied) ovaries ($n = 5$);
2.1 Active: Laying hens with active ovaries in the stage before ovulation, after oviposition ($n = 5$);
2.2 Soft egg shell: Birds with active ovaries, egg in the magnum ($n = 5$);
2.3 Calcified egg shell: Birds with active ovaries, egg in the shell gland ($n = 5$);
2.4 Hard shell: Birds with active ovaries, egg in the shell gland immediately before oviposition ($n = 5$).

The birds were killed by bleeding; EDTA (0.2 mol/l) was used as anticoagulant. The liver tissue was removed immediately after bleeding and was stored at -20 °C until processed.

Red blood cells (RBCs) were separated from the plasma by centrifugation (2500 *g*, 20 min, 4 °C) and for catalase and glutathione-peroxidase determination they were washed twice with cold 0.65% saline. Subsequently, RBCs were haemolysed in a tenfold volume of double-distilled water by freezing and thawing. Intact RBCs were separated from the debris by centrifugation (7000 *g*, 20 min, 4 °C). For the assays, aliquots of the final supernate of the previously separated plasma and haemolysate were used.

Liver tissue was homogenized in a teflon homogenizer in a tenfold volume of ice-cold 0.65% saline. The homogenate was centrifuged (10,000 *g*, 20 min, 4 °C) and the clarified supernate was used for the determinations.

The progesterone and testosterone concentrations of the blood plasma were determined by radioimmunoassay (RIA; Isotope Institute of the Hungarian Academy of Sciences, Budapest), after extraction with diethyl ether. For the determination of 17-β-oestradiol a direct RIA (Serono Diagnostics, Uppsala) developed for human diagnostic purpose was used.

The degree of lipid peroxidation in the blood plasma was determined by using the thiobarbituric acid (TBA) method (Placer et al., 1966), while that of the liver tissue by the method of Uchiyama and Mihara (1978). The quantity of TBA-reactive plasma and tissue products was standardized with 1, 1, 3, 3-tetraethoxy-propane (Fluka, Buchs).

The catalase (E.C.1.11.1.6) activity in the plasma, haemolysate and liver tissue homogenate, was measured kinetically in the presence of hydrogen peroxide as substrate (Beers and Sizer, 1952). The activity was expressed in Bergmeyer units (one Bergmeyer unit is the quantity of catalase that splits 1 g hydrogen peroxide per min at 25 °C).

The glutathione-peroxidase (E.C.1.11.1.9) activity was measured in the plasma, haemolysate and liver tissue homogenate by a direct method in the system of Lawrence and Burk (1976), and the oxidation of reduced glutathione (GSH) was determined with Ellmann's reagent (Serva, Heidelberg; Sedlal and Lindsay, 1968). In the reaction, cumene-hydroperoxide (Schuchardt, Munich) was used as oxidizing agent. The glutathione-peroxidase activity was expressed in units (one unit is the enzyme quantity that oxidizes 1 nmol GSH per min in the above system at 25 °C).

Enzyme activities were expressed for the 10,000-*g* supernate protein content of 1 g plasma, haemolysate and liver tissue homogenate. Protein determinations were performed by the biuret method.

The vitamin E content of the blood plasma was measured by the method of Bieri (1964), while that of the liver by the method of Haacker (1976). For the statistical evaluation of changes between the different stages (1 versus 2.1, 2.1 versus 2.2) Student's *t* test was used.

Results

During egg shell formation the progesterone concentration of the blood plasma significantly increased between stages 2.2 and 2.3; subsequently it underwent a significant decrease between stages 2.3 and 2.4. In group 1 (birds with inactive ovaries) very high individual variability was observed.

Table I

Plasma hormone levels and vitamin E and lipid peroxide status of plasma and haemolysates

Group		Progesterone (nmol/l)	Testosterone (nmol/l)	17-β-oestradiol (μmol/l)	Vitamin E (nmol/l)	TBA-reactive plasma products (MDA) (nmol/ml)	Catalase		Glutathione-peroxidase	
							Plasma (B.U./g protein)	R B C	Plasma (U/g protein)	R B C
1	$\bar{x}$	5.08	2.44	0.12	0.28	10.74	1.12	59.03	4.99	9.26
	$s^{\pm}$	3.01	0.47	0.09	0.04	1.35	0.10	6.75	0.69	4.30
2.1	$\bar{x}$	1.20*	1.81	2.76***	0.41**	9.31	1.18	57.13	4.21	9.79
	$s^{\pm}$	0.47	0.69	0.55	0.04	0.48	0.19	28.42	0.94	2.14
2.2	$\bar{x}$	0.67	1.09	4.27*	0.35	9.24	1.33	45.81	4.06	10.15
	$s^{\pm}$	0.23	0.24	0.97	0.06	0.88	0.20	4.94	1.80	1.14
2.3	$\bar{x}$	42.30**	1.56	3.31	0.37	9.00	1.53	91.58**	5.69	16.93**
	$s^{\pm}$	21.11	0.67	1.22	0.03	1.09	0.20	21.77	1.32	3.70
2.4	$\bar{x}$	4.39**	1.70	3.11	0.36	8.97	2.01	36.92**	2.67	9.42**
	$s^{\pm}$	3.41	0.65	1.95	0.03	0.78	0.41	4.97	1.45	1.72

Significance values: * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.

Table II

Vitamin E and lipid-peroxide status of liver tissue

Group		TBA-reactive products (MDA μmol/g)	Catalase (B.U./g protein)	Glutathione-peroxidase (U/g protein)	Vitamin E (μmol/g)
1	$\bar{x}$	1.44	291.49	68.66	0.062
	$s^{\pm}$	0.67	44.09	10.20	0.047
2.1	$\bar{x}$	0.47	414.93	124.77**	0.122
	$s^{\pm}$	0.21	139.95	33.02	0.093
2.2	$\bar{x}$	1.21*	242.87	40.29**	0.102
	$s^{\pm}$	0.18	19.15	12.17	0.047
2.3	$\bar{x}$	1.27	436.43*	65.23*	0.062
	$s^{\pm}$	0.62	100.28	6.63	0.043
2.4	$\bar{x}$	0.32	394.45	73.26	0.103
	$s^{\pm}$	0.16	54.49	15.23	0.031

Significance values: * = $P < 0.05$; ** = $P < 0.01$.

The 17-β-oestradiol content of the blood plasma significantly increased between stages 1 and 2.1, and also between stages 2.1 and 2.2. Although, due to the use of the direct RIA, the obtained values are higher than the mean values reported in the literature, the tendency can be observed.

No mathematically demonstrable difference existed between the stages in the testosterone concentration of the blood plasma.

No substantial change occurred in the quantity of TBA-reactive substances of the blood plasma during egg shell formation.

The catalase activity of the plasma increased considerably in group 2.4; it should, however, be noted that in the "active" stages 2.1–2.4 a continuous increase was observed.

The glutathione-peroxidase activity of the blood plasma showed a notable change in the second phase of egg shell formation (groups 2.3–2.4). In group 2.3, glutathione-peroxidase activity showed a significant increase as compared to group 2.2, then decreased.

In the vitamin E concentration of the blood plasma, a pronounced difference was observed between the "active" (group 2) and "inactive" (group 1) stages, while there were no statistically demonstrable differences between the "active" groups (2.1–2.4).

The catalase activity of RBCs (1 : 10 haemolysate) showed a characteristic peak during egg shell formation; in group 2.3 it was characterized by a considerable increase, whereas in group 2.4 by a marked decrease.

The glutathione-peroxidase activity of the haemolysate underwent changes similar to those in the catalase activity.

The quantity of TBA-reactive products in the liver tissue showed a marked increase between the "inactive" and "active" groups.

In the catalase activity of the liver tissue (10,000 *g* supernate) there was a significant increase between stages 2.3 and 2.4. Changes in the glutathione-peroxidase activity of the liver tissue followed a characteristic cycle pattern which corresponded to reproductive-biological changes. Between stages 1 and 2.1 a marked increase, between stages 2.1 and 2.2 a pronounced decrease, while between stages 2.2 and 2.3 again an increase, was demonstrated.

No substantial changes occurred in the vitamin E content of the liver tissue during egg shell formation. Similarly, no difference existed between the "active" and "inactive" groups in this respect.

Discussion

The results of the present study indicate that the vitamin E content of the blood plasma was considerably higher in the "active" groups (hens with active ovaries) than in the "inactive" group, a finding consistent with the relationships

described previously between the vitamin E content of the feed and that of the egg (Scott et al., 1976). Namely, only the vitamin E taken up by the birds with the feed and transported from the tissues of storage to the egg by the blood stream can be incorporated into the egg, more precisely, into the yolk. The hormonal changes have an effect also on the distribution of vitamin E within the organism, since simultaneously with nearly identical vitamin E concentrations in the liver tissue a considerable increase of vitamin E concentration was found in the blood plasma. When compared on a quantitative basis, however, the changes observed in the blood plasma cannot be demonstrated for the whole liver.

The liver has an important role in the formation of yolk material; the high energy requirement of egg formation increases the oxygen uptake of tissues, among others liver tissue, and oxygen dissolves in lipids eight times better than in water (De Duve and Hayaishi, 1978). The quantity of malondialdehyde (TBA-reactive products) was considerably higher in the "active" group as compared to birds with inactive ovaries, suggesting that in the liver tissue there is an enhanced lipid-peroxidation in the period of egg production. This effect might be explained by hormonal changes, primarily with alterations of the 17-β-oestradiol level, for in mammals the hydrogen peroxide concentration and peroxidase activity of the uterus were found to increase upon the effect of oestrogens (Katz et al., 1982). However, no data are available on similar effects of oestrogens on the reproductive organs and liver tissue of birds.

No substantial changes occurred in the malondialdehyde content of the blood plasma. This finding indicates that the liver tissue eliminates the produced aldehydes, ketones and free-radicals.

Changes of the catalase activity of the blood plasma and liver tissue followed a characteristic cycle pattern during egg shell formation. The observed changes are related to alterations in the oestrogen content of the blood plasma. After oestrogen peaks a marked increase occurred in the catalase activity of liver tissue, indicating free-radical formation due to hormone-induced changes in intermediary metabolism.

The changes of glutathione-peroxidase activity of the liver tissue were closely related to fluctuations of the plasma 17-β-oestradiol level. These changes indicated that oestrogen and progesterone influenced, besides other parameters, the vitamin E and lipid-peroxide status of the organism. Of the hormonal changes, the alteration of the 17-β-oestradiol concentration was consistent with changes observed in the above parameters. An interpretation of the changes observed in enzyme activities cannot be based on the present results; i.e. it cannot be decided whether there is *de novo* protein synthesis or only activation of the "ready" enzyme molecules. Further experiments are needed to elucidate this aspect. As regards the blood plasma and RBCs, the results show that although the primary target of the overload constituted by hormonal changes is

the liver tissue, this effect manifests itself also at the level of the blood plasma. Changes in the enzyme activity of RBCs support the idea that the hormone-induced change is an enzymatic adaptation realized through activation of molecules. Namely, no notable protein synthesis can be expected here.

The present results indicate that in high-producing laying hens great attention should be paid to vitamin E supply and also to the proper lipid composition and peroxide content of the feed.

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EFFECT OF CAECOTROPHY ON THE NITROGEN METABOLISM OF ANGORA RABBITS

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In two experiments conducted with mature, producing angora rabbits the changes occurring in the digestibility of nutrients due to the prevention of caecotrophy were studied at a lower (12.8%) and a higher (16.7%) protein level. The nitrogen metabolism of the rabbits was followed. Furthermore, it was examined whether the protein-rich feed altered the effect of caecotrophy. Eight rabbits were used in each experiment; four of them were wearing a rigid plastic collar to prevent caecotrophy (experimental rabbits).

The digestibility of nutrients, with the exception of crude protein, was not significantly better in the control rabbits kept on the protein-rich diet than in those kept on low-protein diet. The virtual digestibility of crude protein was 82.1 and 75.2%, respectively ($P < 0.001$). Prevention of caecotrophy worsened the digestibility of nutrients, with the exception of crude fibre. The worsening in digestibility was significant at both protein levels only for crude protein. At the higher protein level (16.7%), digestibility of N-free extracts was also significantly higher for the experimental animals than for the controls.

Studies on N metabolism revealed that the experimental (i.e. collar-wearing) rabbits excreted significantly more N in the faeces daily than the control ones. In the urine, the control rabbits excreted more N.

At the lower protein level (12.8%), the N retention of the control animals was significantly higher than that of the experimental ones. The difference was 0.47 g N/day/animal, which is 17% of the total N intake and corresponds to 2.6 g protein. At the higher protein level the N retention of the control animals was not significantly higher than that of the experimental ones; the difference was 0.29 g N/day/animal, which is 8.4% of the total N intake and corresponds to 1.8 g crude protein.

Keywords. Caecotrophy, nitrogen metabolism, nutrient digestibility, angora rabbit.

Caecotrophy plays an important role in the digestion and internal metabolism of the rabbit. "Caecotrophy" means that the rabbit consumes directly from its anus the characteristic faecal pellets appearing there from time to time. This material originates from the caecum, has specific composition, and consists, besides the caecal contents subjected to microbial activity, of considerable numbers of live bacteria (Hörnicke, 1972). The soft faeces (pH 6.0) does not mix immediately with the stomach contents; it remains in one mass along the greater curvature. Despite the low gastric pH (1.0–1.5), the mucous cover settled on the mass is maintained for 6–8 h, due to the high phosphate buffer concentration present in it. In each pellet the bacterial fermentation of carbohydrates to lactic acid continues until the mucous cover is disrupted and the contents of the pellet get into the process of digestion. Alexander and Chowdhury (1958), Griffiths and Davies (1963) and Hörnicke and Mackiewicz (1977) demonstrated

in the rabbit stomach the secretion of a bacteriolytic substance, which is activated by pepsin. This substance attacks the bacteria released from the pellets, rendering possible the degradation of bacterial proteins by proteolytic enzymes in the intestine.

According to Villard and Raynaud (1968), the rabbit consumes considerable amounts of faeces, generally taking up 50 to 80% of the total faecal output. The importance of caecotrophy in protein and dry-matter digestion and in the improvement of digestibility was recognized very early (Huang et al., 1954; Thacker and Brandt, 1955). The quantity of total and essential amino acids is higher in the soft faeces than in the feed consumed. The increased amino acid content in the soft faeces was explained by the activity of microorganisms present in the caecum (Proto and Gianani, 1969; Yoshida et al., 1971). Furthermore, it has been shown that in the caecum certain amino acids undergo deamination and that new essential amino acids may be synthesized (Marty and Raynaud, 1963). The essential amino acids derived from caecotrophy make possible for the adult rabbit to live on feeds containing poor-quality protein. The biochemistry of caecal fermentation is roughly known (Marty et al., 1973), but the factors influencing the fermentation and its rate (e.g. the effects of certain feeds) have been little studied so far. According to Proto (1976), in mature rabbits the protein synthesized by microorganisms is a good supplementation of the feed proteins. However, few data are available on the degree to which the above process contributes to meeting the amino acid requirements of the rabbit. Adamson and Fisher (1971) and Davidson and Spreadbury (1973) furnished data on the essential amino acid requirement of meat rabbits. For angora rabbits such data are scarce, and are restricted to sulphur-bearing amino acid requirements (Hoffmann–La Roche, 1980).

To determine the degree and effects of preventing caecotrophy, we subjected the mixed faeces and mixed urine samples of control and experimental (collared) rabbits to chemical analyses, in an attempt to obtain information on the effect of caecotrophy on the biological value of different nutrients. The purpose of the present investigations was to determine the degree of changes induced by prevention of caecotrophy in the digestibility of certain nutrients, and to monitor the N metabolism of rabbits. Furthermore, we examined whether there were changes in the effect of the prevention of caecotrophy when a feed of higher protein content was fed.

Materials and methods

Two experiments were conducted; in experiment A the feed contained 12.8%, while in experiment B it contained 16.7%, protein.

1.5-year-old angora buck-rabbits of 3318 g (s $\pm$ 67 g) body mass, registered for "Medima 98" utilization, were used. The rabbits were accommodated in

individual feed-utilization cages. Eight rabbits, allotted to two groups, took part in each experiment. Rabbits of the first group (n = 4; hereinafter referred to as "experimental" rabbits) were fitted up with a rigid plastic collar to prevent them from consuming soft faeces. The experiment consisted of a 7-day pre-feeding and a 5-day experimental period. The experimental rabbits wore the collar for 8 days (for 3 days in the pre-feeding period and 5 days in the experimental period).

The composition of the diets is shown in Table I. For experiment B, the protein content of the feed fed in experiment A was raised by adding extracted sunflower groats.

The nutrient content of the experimental diets is shown in Table II. Between diets fed in experiment A and B, substantial difference existed only in crude protein concentration (12.8% versus 16.7%).

Parallel and nearly proportionally with the rise of crude protein concentration, the amount of amino acids in the feed also increased (Table III). The diet was fed in a single daily dose of 120 g. The faeces as well as the urine of

Table I

Composition of mixed feed

Feed components %	Experiment A	Experiment B
Corn	15.0	12.8
Wheat	9.0	7.7
Barley	9.0	7.7
Oat	3.0	2.6
Wheat bran	2.0	1.7
Sunflower groats	*5.0*	*20.0*
Lucerne hay	27.0	22.0
Sugar-beet slices	5.0	4.3
Corn-cob	18.0	15.3
Straw meal	5.5	4.7
Salt (NaCl)	0.5	0.4
Feed lime	0.5	0.4
Premix	0.5	0.4
	100.0	100.0

Table II

Chemical composition of experimental diets

Nutrients	Experiment A	Experiment B
	%	%
Dry matter	90.14	90.83
Crude ash	5.00	5.03
Crude protein	12.85	16.75
Crude fibre	16.10	14.84
Crude fat	2.39	2.56
N-free extracts (N. F. E.)	53.80	51.65

Table III

Amino acid contents of feed, expressed as % of dry matter

Amino acids	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Cys	Val
Experiment A	1.04	0.49	0.55	1.71	0.84	0.56	0.58	0.22	0.66
Experiment B	1.35	0.61	0.68	2.29	0.94	0.77	0.76	0.27	0.87
Amino acids	**Met**	**Ile**	**Leu**	**Tyr**	**Phe**	**Lys**	**His**	**Arg**	**Try**
Experiment A	0.31	0.52	0.95	0.58	0.58	0.52	0.27	0.67	0.11
Experiment B	0.38	0.67	1.16	0.67	0.77	0.63	0.36	1.01	0.20

the rabbits was collected throughout the 5-day experimental period. The N content of the freshly-excreted faeces and urine was determined daily by the Kjeldahl method. Other nutrients included in the utilization experiment were determined in dried faeces, together for the 5-day experimental period but separately for each animal, according to the Hungarian standard MSz 6830-66.

Results and discussion

Effect of protein concentration on the digestibility of the various nutrients in control rabbits

The changes brought about by increasing the protein content of the feed in the digestibility of the various nutrients were monitored in a feed utilization experiment (Table IV). In experiment B (16.7% protein), the apparent (virtual) digestibility of dry matter, crude protein, crude fat and ash tended to increase. The improvement of digestibility was not significant, with the exception of crude protein, the digestibility of which increased from 75.2% to 82.1% ($P < 0.001$). No change occurred in the digestibility of the crude fibre, while the digestibility of N-free extracts tended to worsen (by 0.9%). Glover and

Table IV

Digestibility (%) of nutrients in rabbits kept on low- or high-protein diet under conventional conditions

	Experiment A (12.8% protein)	Experiment B (16.7% protein)
	Control	Control
Dry matter	66.3 ± 7.9	68.4 ± 5.0
Crude protein	75.2 ± 6.5	82.1 ± 4.0
Crude fibre	31.0 ± 12.0	29.1 ± 9.2
Crude fat	70.9 ± 9.2	71.9 ± 4.0
N-free extracts	70.3 ± 6.7	69.4 ± 3.4
Ash	55.7 ± 12.0	56.1 ± 9.4

Duthie (1958) and Aguilera (1973) showed that the digestibility of crude protein improved if the feed protein level was raised. According to Eriksson (1952) the increased feed protein quantity has an advantageous effect on the digestibility of not only the protein but also on that of the other nutrients. It should, however, be emphasized that the digestibility in this sense is a virtual digestibility since we cannot tell how large proportions of the proteins present in the faeces originated from mucosal epithelial cells detached from the intestinal wall, from gastric (digestive) juices, from microorganisms present in the intestine, or from post-digestion remnants of the feed.

Effect of the prevention of caecotrophy on the digestibility of nutrients

This question was studied by comparing the digestibility per cent obtained for the experimental and control rabbits. In experiment A, poorer digestibilities were obtained for all nutrients except crude fibre for the collar-wearing rabbits than for the control animals (Table V). Protein digestibility was significantly better in the control animals (75.2%) than in the experimental ones (62.3%) ($P < 0.001$). The digestibility of crude fat was by 3.4%, while that of the N-free extracts by 3.9%, higher than in the control rabbits (the differences were not significant). In experiment B (Table VI), the same tendency was observed for the changes of nutrient digestibility as in experiment A. Here also

Table V

The effect of prevention of caecotrophy on the digestibility of nutrients, in %

	Experiment A	
	Experimental rabbits	Control rabbits
Dry matter	65.1 ± 5.9	66.3 ± 7.9
Crude protein	62.3 ± 6.9	75.2 ± 6.5
Crude fibre	30.9 ± 8.4	31.0 ± 12.0
Crude fat	67.5 ± 10.8	70.9 ± 9.2
N-free extracts	66.4 ± 6.0	70.3 ± 6.7
Ash	50.4 ± 7.5	55.7 ± 12.0

Table VI

The effect of prevention of caecotrophy on the digestibility of nutrients, in %

	Experiment B	
	Experimental rabbits	Control rabbits
Dry matter	67.9 ± 6.6	68.4 ± 5.0
Crude protein	71.4 ± 6.8	82.1 ± 4.0
Crude fibre	29.8 ± 7.0	29.1 ± 9.2
Crude fat	69.2 ± 6.5	71.9 ± 4.0
N-free extracts	61.2 ± 6.5	69.4 ± 3.4
Ash	54.9 ± 8.1	56.1 ± 8.4

a considerably better protein digestibility was obtained for the control rabbits (82.1%) than for the experimental ones (71.4%). However, here the difference is only 10.7% as opposed to the 12.9% found in experiment A, where the rabbits consumed less protein. The difference is highly significant ($P < 0.001$). Although the difference between the protein digestibility % obtained in experiment A and B is not significant, it suggests that the rabbits wearing collar are more dependent on the quantity and quality of protein given in the feed. In this experiment the digestibility of N-free extracts was by 8.2% higher in the control animals ($P < 0.01$). Contrarily, Frage and De Blas (1977) found that caecotrophy had no significant influence on the digestibility of nutrients serving as energy source.

The question arises whether it is possible to compare the digestion of experimental (collared) and control (conventionally kept) rabbits. Yoshida et al. (1971) showed that the stress constituted by fitting up the rabbits with a collar to prevent caecotrophy did not cause any significant change in the composition of either the solid or the soft faeces. Proto et al. (1968) and Stephens (1976) found that if a good-quality feed was offered, collar-wearing constituted a greater stress for the animals than the slight decrease of nutrient digestibility. Our results do not contradict this statement.

Due to the prevention of caecotrophy, the digestibility of nutrients underwent a negligible decrease in both of the present experiments. In our opinion, the significantly higher digestibility of N-free extracts observed for the control rabbits in experiment B was the consequence of the increased protein content of the feed. This higher protein content must have forced the rabbits to utilize energy better. The cause of improved protein digestibility is discussed in the following.

Studies on nitrogen metabolism

In experiment A (Table VII), the experimental rabbits took up 2.13 g, while the controls 2.22 g, N from the feed. The stress constituted by fitting up the rabbits with a collar resulted in a slight (4.5%) decrease of feed consumption and N intake. The N quantity present in the faeces excreted by the experimental animals in one day's time was 0.80, while in that of the controls 0.55 g. The difference (0.25 ± 0.04 g/rabbit/day) is significant. Thus, in addition to the N taken up from the feed, the control rabbits took up an extra 0.25 g N from the soft faeces; their overall N uptake was 2.47 g (by 15.7% more than that of the experimental rabbits). In the experimental animals the quantity of absorbed N coming from the digestible protein of the feed was 1.33 g/day (62.4%), whereas in the control ones 1.92 g/day (77.7%), 0.59 g more than in the experimental rabbits. This was due to the increased N uptake on the one hand and caecotrophy on the other.

According to Yoshida et al. (1968), the microorganisms of the digestive tract are able to improve the biological value of proteins present in the soft faeces which the rabbit can utilize through caecotrophy. This increase in the biological value of the soft faeces proteins can be traced back not only to microbial activity but also to the considerable number of mucosal epithelial cells detached from the intestinal wall and to the secretion of digestive juices in the intestinal tract as well (Marty and Raynaud, 1963). Yoshida (1971) found that the quality of proteins present in the soft faeces of rabbits was superior to that of the feed proteins. Therefore, it was expected that the control animals utilize the additional N obtained through caecotrophy better (as a biologically more valuable protein) than the experimental rabbits the biologically less valuable proteins of the feed. Contrarily, the experimental animals excreted less N (0.86 g) daily in the urine than the controls (1.03 g). The difference is not significant but is suggestive of the adaptability of the organism to compensate for a protein supply of poorer quality and lesser quantity. The excess urinary excretion of N observed for the control rabbits allows us to conclude that the utilization of the surplus protein originating from the consumed soft faeces is worse than that of the feed proteins. In the opinion of Yoshida et al. (1971) this question could be elucidated by feeding soft faeces to germfree and conventional rabbits. However, germfree rabbits do not consume their soft faeces.

In spite of their higher urinary N excretion, the N retention of the control rabbits (0.89 g) exceeded that of the collar-wearing rabbits (0.47 g). The difference is 0.42 g/day/animal and is highly significant ($P < 0.001$). This corresponds to 2.6 g protein and is 17.0% of the consumed N.

In experiment B (Table VIII) the experimental animals took up 3.03 while the controls 3.22 g (6.2% more) N from the feed. In the experimental rabbits, the faeces excreted daily contained 0.82 g, while that of the controls significantly ($P < 0.01$) less, only 0.58 g N (the difference being 0.24 ± 0.11 g).

Table VII

Results of the nitrogen balance

		Experiment A			
		Experimental rabbits (n = 4)		Control rabbits (n = 4)	
		g	%	g	%
N taken up with the feed,	g/animal/day	2.13 ± 0.34	100	2.22 ± 0.31	104.5
N excreted in the faeces,		0.80 ± 0.17	37.5	0.55 ± 0.18	24.7
N taken up from the faeces,		—	—	0.25 ± 0.04	11.2
Total N intake,		2.13 ± 0.34	100	2.47 ± 0.33	115.7
N absorbed after digestion,		1.33 ± 0.21	62.4	1.92 ± 0.24	77.7
N excreted in the urine,		0.86 ± 0.22	40.4	1.03 ± 0.32	41.7
N retention,		0.47 ± 0.12	22.0	0.89 ± 0.22	36.0

Table VIII

Results of the nitrogen balance

		Experiment B			
		Experimental rabbits (n = 4)		Control rabbits (n = 4)	
		g	%	g	%
N taken up with the feed,	g/animal/day	3.03 ± 0.27	100	3.22 ± 0.29	106.2
N excreted in the faeces,		0.82 ± 0.23	27.5	0.58 ± 0.12	18.0
N taken up from the faeces,		—	—	0.24 ± 0.11	7.4
Total N intake,		3.03 ± 0.27	100	3.46 ± 0.27	113.6
N absorbed after digestion,		2.21 ± 0.23	72.9	2.88 ± 0.29	82.9
N excreted in the urine,		1.35 ± 0.29	44.5	1.73 ± 0.31	50.0
N retention,		0.86 ± 0.26	28.4	1.15 ± 0.38	33.2

The control rabbits took up from the faeces the same amount of N as in experiment A. Their total N intake was 3.46 g/animal/day, by 13.6% more than that of the experimental animals. For the experimental rabbits, the quantity of absorbed N coming from the digestible proteins of the feed was 2.21 g (72.9%), whereas in the control rabbits 2.88 g (82.9%) of the total protein uptake was absorbed (which is 0.67 g more than that obtained for the experimental animals). The control rabbits excreted more (1.73 g) N in the urine than the collar-wearing ones (1.35 g); the difference is not significant.

N retention was 1.15 g/day in the control and 0.86 g in the experimental group. The difference, corresponding to 1.8 g protein, is not significant.

Conclusions

The N quantity taken up from the soft faeces was identical in angora rabbits given either a lower (12.8%) or a higher (16.7%) protein diet, 120 g/day.

A low-protein diet resulted in a 15.9% worse protein digestibility (experiment A) in the rabbits prevented from practising caecotrophy (experimental rabbits) as compared to conventionally kept (control) rabbits.

When feeding a diet of higher protein content (16.7%), the difference between the two groups in the protein digestibility coefficient was only 10% (experiment B).

In both experiments the quantity of N excreted in the urine was higher in the control rabbits than in the experimental ones. The difference appeared to be more pronounced in experiment B; however, it was not significant in either case.

At a lower protein level, caecotrophy resulted in a significantly higher N retention in the control animals as compared to the experimental ones. In ex-

periment B also, the control rabbits showed a higher N retention than the experimental ones; however, the difference between the two groups in N retention was not significant.

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EFFECT OF CAECOTROPHY ON PROTEIN AND AMINO ACID METABOLISM OF ANGORA RABBITS

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Experiments were conducted with angora rabbits to determine how the consumption of high- and low-protein diets and prevention of caecotrophy influenced the protein and amino acid metabolism. Caecotrophy was prevented by a plastic collar.

The daily feed consumption and the quantity of excreted faeces were weighed, and the nitrogen and amino acid contents of the collected faeces were determined.

The protein, amino acid and urea concentrations were determined in blood samples taken from the rabbits.

The faecal analyses revealed that, in addition to the protein content of the feed, prevention of caecotrophy also influenced the nitrogen and amino acid excretion.

In the control animals kept as usual 75 to 81%, whereas in the collar-wearing (experimental) rabbits about 67 to 71%, of the total N excreted in the faeces was amino acid nitrogen.

In the control animals, the quantity of excreted amino acids calculated for identical faecal N amounts (mg amino acid/g N) exceeded that found for the experimental animals by 12.5% in both experiments (A and B). When feeding low-protein diets, the majority of the excreted amino acids consisted of non-essential, while for high protein diets of essential, amino acids.

The results of blood analyses suggest that the quantity of amino acids in the blood plasma can be an indicator of the animals' amino-acid supply status.

The significantly better blood parameters (protein, total amino acid N, urea) obtained for the control rabbits *practising caecotrophy* and fed a low-protein diet (C rabbits) indicate a higher protein and amino acid utilization. In rabbits fed a diet of higher protein content, the effect of caecotrophy was not significant.

Keywords. Caecotrophy, protein metabolism, amino acid metabolism, angora rabbit.

Caecotrophy plays an important role in the nutrition and digestive physiology of the rabbit. Since the end of the last century several authors (Moroth, 1882; Lang, 1981) have dealt with caecotrophy; however, its complex mechanism and effect on internal metabolic processes have not been fully elucidated yet.

In the present work the effect of prevention of caecotrophy on the amino acid metabolism of angora rabbits and the changes occurring in some other metabolic parameters of rabbits fed low- and high-protein diets were investigated.

Materials and methods

Two series of experiments were conducted. In experiment A the rabbits were fed a diet of lower (12.8%), while in experiment B higher (16.8%), protein content. In both series of experiments both the control (group C; rabbits practising caecotrophy) and the experimental (group E; rabbits prevented from practising caecotrophy) groups comprised 4 adult angora rabbits designated "Medima 98"; 16 rabbits were used altogether. The animals were kept individually in metabolic cages.

The quantity of the consumed feed and excreted faeces was recorded daily, and the nitrogen (N) and the amino-acids were determined in the collected faecal samples.

In both experiments blood samples were taken from the ear vein on the 5th day of feeding, always at the same time, before the morning feeding.

The amino acids were determined in the feed, faecal and blood samples in an automatic amino-acid analyser (type BC-200). The blood samples were deproteinized before assayed (Stein and Moore, 1954).

The protein content of the blood plasma was determined by the biuret method (Bálint, 1962), and its free amino-acid N content according to Folin and Danielson.

The urea concentration of the blood plasma was measured by the Berthelot reaction (Klinisches Lab. Merck, 1974).

For further methods, see the accompanying paper (Teleki et al., 1985; p. 41 in the present issue).

Results

The results of both experiments (A and B) are summarized in Table I.

In experiment A, the average daily N excretion with the faeces was 0.55 g N for group C and 0.79 g N for group E. Of the total N excreted, 0.45 g N and 0.53 g N was amino-acid N in group C and group E, respectively; thus, 81 and 67% of the total faecal N was amino-acid N.

In experiment B, the average daily N excretion with the faeces of the control rabbits was 0.57 g, of which 0.43 g was amino-acid N. In the experimental group of rabbits the average daily N excretion with the faeces was 0.82 g, of which 0.59 g was amino-acid N. In this experiment, 75 and 71% of the excreted total N was amino-acid N in the C and E animals, respectively.

The relative amino acid surplus measured in the faeces of groups C is best expressed by the amino acid quantity calculated for 1 g excreted N. Table II indicates that in both experiments the total amino acid content (expressed in mg/g N) of the faeces of control rabbits (C) exceeded on the average by 12.5% that of the experimental animals (E). In experiment A, all amino acids, except

Table I

Daily faecal N and amino-acid N excretion of experimental rabbits, g/day/animal

Groups	Experiment A		Experiment B	
	Control (C)	Experimental (E) (collared)	Control (C)	Experimental (E) (collared)
N excretion	0.55	0.79	0.57	0.82
Amino-acid N excretion	0.45	0.53	0.43	0.59
Amino acid N, in % of total N	81	67	75	71

N = nitrogen.

Table II

Amino acid contents of the faeces, mg/g N

Amino acids	Experiment A		Experiment B	
	Control	Experimental	Control	Experimental
Aspartic acid	518 ± 84	480 ± 57	355 ± 34	490 ± 123
Threonine	300 ± 13	217 ± 10	282 ± 77	258 ± 12
Serine	282 ± 26	191 ± 11	282 ± 66	211 ± 16
Glutamic acid	809 ± 96	947 ± 86	627 ± 130	688 ± 83
Proline	318 ± 55	303 ± 44	300 ± 33	264 ± 9
Glycine	300 ± 23	204 ± 8	264 ± 67	199 ± 65
Alanine	363 ± 15	263 ± 16	436 ± 58	278 ± 52
Cystine	182 ± 74	138 ± 74	173 ± 52	126 ± 57
Valine	236 ± 26	256 ± 21	300 ± 29	212 ± 103
Methionine	181 ± 23	125 ± 50	273 ± 25	119 ± 70
Isoleucine	181 ± 30	151 ± 29	209 ± 33	172 ± 81
Leucine	418 ± 37	342 ± 67	400 ± 50	325 ± 92
Tyrosine	218 ± 25	164 ± 15	173 ± 17	152 ± 81
Phenylalanine	182 ± 41	171 ± 17	209 ± 26	198 ± 41
Lysine	309 ± 39	257 ± 44	300 ± 65	265 ± 35
Histidine	181 ± 19	151 ± 35	155 ± 40	119 ± 34
Arginine	227 ± 24	211 ± 31	173 ± 16	212 ± 42
Total amino acid	5205	4571	4911	4288

glutamic acid and valine, were present in higher concentrations in the faeces of the C rabbits than in that of the E rabbits, whereas in experiment B the same held true for all amino acids except glycine, glutamic acid and arginine.

In both experiments the faeces of the C rabbits was considerably more abundant in methionine, cystine, leucine, lysine, threonine, alanine, glycine and serine than the faeces of the E rabbits.

Table III shows the distribution of essential and non-essential amino acids in the faeces of the rabbits, expressed in mg/g N. In both groups of experiment A the faeces contained more non-essential amino acids, whereas in the faeces of rabbits of experiment B the quantity of essential amino acids was higher. Table

Table III

Distribution of amino acids in the faeces

	Experiment A		Experiment B	
	Control rabbits	Experimental rabbits	Control rabbits	Experimental rabbits
	mg/g N			
Total amino acid content	5.21	4.57	4.91	4.29
Essential amino acid content	2.40	2.02	2.76	2.22
Non-essential amino acid content	2.81	2.55	2.15	2.07
	%			
Essential/Total amino acids	46.1	44.2	56.2	51.8
Non-essential/Total amino acids	53.9	55.8	43.8	48.2

Table IV

Blood plasma parameters of the rabbits

	Experiment A		Experiment B	
	Control rabbits	Experimental rabbits	Control rabbits	Experimental rabbits
Total protein, g/l	71.7 ± 6.9	57.7 ± 10.8	68.6 ± 9.3	60.2 ± 3.3
Urea, mmol/l	7.1 ± 0.7	9.6 ± 0.9	8.4 ± 1.3	9.3 ± 1.3
Amino acid N, mmol/l	8.3 ± 1.2	5.9 ± 0.3	8.9 ± 0.8	8.3 ± 1.1

III shows also the percentile distribution of amino acids. In group E of experiment A, 44.2%, while in the control rabbits of the same experiment 46.1% of the faecal amino acids was constituted by essential amino acids. In both groups of experiment B the rabbits excreted more essential than non-essential amino acids; in the faeces of experimental rabbits 51.8, whereas in that of the controls 56.2% of the total amino acid content was essential amino acid.

The free amino acid content of blood plasma samples withdrawn at the end of the experiments was determined, since this parameter indicates the amino acid supply status of the rabbits. Figure 1 shows the quantitative data for

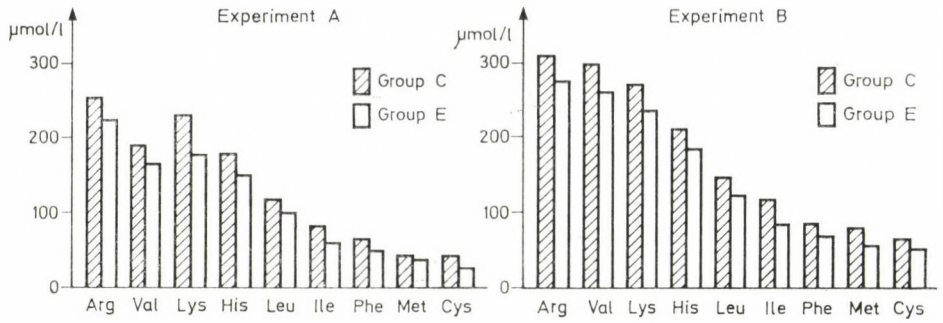

Fig. 1. Essential amino acid contents of the blood plasma in experiments A and B

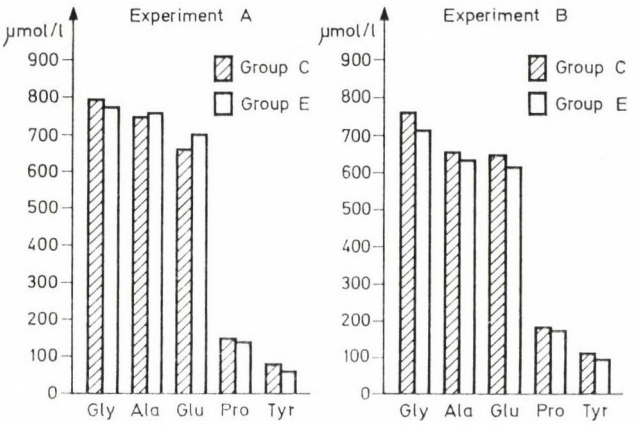

Fig. 2. Non-essential amino acid contents of the blood plasma in experiments A and B

the measured essential amino acids in the E and C groups of both experiments. In both experiments arginine was present in the highest, while methionine and cystine in the lowest concentrations in the blood plasma. In experiment A the blood plasma contained low concentrations of valine both in experimental and control rabbits; furthermore, all essential amino acids were present in lower concentrations than in the blood plasma of the rabbits used in the second experiment (experiment B). The blood plasma of control rabbits was relatively rich in essential amino acids in both experiments.

Figure 2 shows the quantity of non-essential amino acids for both experiments, in the order of decreasing concentration. Of the non-essential amino acids glycine was present in the highest, while tyrosine in the lowest, concentration in the blood of all animals. In experiment A the quantity of non-essential amino acids in the E rabbits was slightly higher, whereas in experiment B lower, than in the controls.

Some other blood plasma parameters indicative of protein and amino acid supply status and utilization were also determined. In experiment A there were large differences between the E and C rabbits in the total protein, urea, and total amino acid content of the blood plasma (Table IV). There was a highly significant ($P < 0.001$) difference in all the three parameters between the two groups, in favour of group C. In experiment B there were no significant differences between the two groups in the above parameters ($P > 0.05$).

Discussion

According to the results of the present experiments, the protein and amino acid metabolism of the rabbit is significantly influenced, in addition to the quantity and quality of dietary protein, by caecotrophy.

At both dietary protein levels the control rabbits excreted more amino acid N in the faeces than the collar-wearing rabbits. Seventy-five to 83% of the excreted total faecal N was amino-acid N in the C, while 67 to 71% in the E group.

It can be, therefore, stated that both the quantity and the quality of the excreted faecal N was influenced by caecotrophy. The experimental rabbits excreted nearly identically less amino-acid N (61 and 71%) at both dietary protein levels, while the control animals excreted, within their lower N excretion, more amino-acid N in both experiments.

At both dietary protein levels, in the faeces of control rabbits the quantity of excreted amino acids calculated for the same faecal N (mg amino acid/g N) exceeded that found for the experimental animals by 12.5% on the average (Table I).

At the lower dietary protein level non-essential amino acids were present in a slightly higher quantity in the faeces of both the E and the C group (53.9 vs. 55.8%), while at the higher protein level the excretion of essential amino acids increased (51.8 vs. 56.2%).

Both the protein and amino acid content of the consumed feed and caecotrophy were found to influence the N content and amino acid composition of the faeces, and thus also the utilization of N and amino acids, a fact that had been referred to by Thacker and Brandt (1955) and Yoshida et al. (1971).

The amino acid concentrations of the blood can be good indicators of essential amino acid supply. In our experiments the essential amino acid content of the blood was higher in rabbits consuming the high-protein feed of higher amino acid content.

In both experiments the essential amino acid content of the blood plasma was higher in group C than in group E; this means that caecotrophy exerted an advantageous influence on the amino acid content of the blood. In their experiments Adamson and Fischer (1976) showed that the blood amino acid levels are good indicators of the amino acid supply status; however, this method cannot be used for determining the excess amino acid supply.

At the lower dietary protein level a highly significant difference was found between the control and experimental groups in the total amino acid N and urea concentration of the blood, while at the higher dietary protein level the difference in these parameters was less expressed. The results of these blood analyses suggest that caecotrophy has a much more efficient influence on protein and amino acid metabolism in rabbits fed diets markedly deficient in essential amino acids than in rabbits fed diets of more valuable protein content, that is, higher essential amino acid content. These results are in good agreement with our observations published in the accompanying paper (Teleki et al., 1985; see p. 41 in the present issue) made on N metabolism and nutrient utilization.

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IN VITRO STUDY OF BIDIRECTIONAL GLYCINE TRANSPORT IN PORCINE SMALL INTESTINE

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The effect of pH, Zn and Na on glycine (Gly) absorption was investigated in vitro, in everted intestinal sacs prepared from porcine jejunum. The 5-cm-long sacs were bathed in Kowarski's chloride solution (Kowarski et al., 1974) of 20 different compositions, containing 29.6–59.2 kBq/ml ^{3}H-Gly and 15 mmol/l nonlabelled Gly, at 37 °C for 60 min, under O_2–CO_2 perfusion. The pH of the solutions was adjusted to 7.4, 6.8 and 5.8, with Zn concentrations of 0, 50 or 200 mg/kg, respectively, with or without Na. The sodium-free media contained 0.3 mol/l mannitol instead of NaCl. In addition to these, Zn-free and Na-containing bathing (mucosal) solutions (pH 6.8) of 0.5 mmol/l 2,4-dinitrophenol (DNP) or 10 mmol/l sodium iodoacetate (NaIA) content were also prepared. In all cases 2 ml Krebs-Ringer-HCO_3 (KR) solution (pH 7.4), containing 18.5–25.9 kBq/ml ^{14}C-Gly and 15 mmol/l nonlabelled Gly was inside the sac (on the serosal side). Transport data were evaluated by analysis of variance (ANOVA) and by a one- or two-sample t test. Correlations and regressions were also calculated.

The Gly transport was sodium-dependent in both directions; this dependence primarily occurred in uphill efflux processes. In the absence of sodium, the efflux from the tissue into the mucosal solution decreased by 40–86% in the different groups, whereas that from the tissue into the serosal solution by 35–77%. Such a decrease could be observed in downhill transport processes towards the tissue as well. The quantity of Gly transported from the mucosal solution into the tissue decreased by 16–48% in the different groups. In the absence of sodium, with the exception of one case, the serosal solution → tissue transport values were also lower, maximum by 68%. However, according to the ANOVA the lack of Na failed to cause a significant difference in the latter process. The interaction between the effects of sodium and pH was significant: on the one hand, the effect of Na^+ decreased at a pH shift to the strong acidic direction; on the other hand, pH exerted an effect on transport only in the presence of Na^+ (it decreased the uphill efflux from the cell in both directions). In the presence of Na^+, the Zn salt reduced the efflux from the tissue towards the serosa. Since maximum one-third of the Gly taken up by the tissue from the mucosal solution was transported into the serosal solution during the 1-h period of study, passage across the basolateral membrane from the tissue towards the serosal solution seemed to be the limiting step of transport. In the experiment where DNP was used instead of mannitol, results of a similar trend and magnitude were obtained. Sodium iodoacetate (NaIA) further decreased the transport. The difference between values obtained in the presence and absence of Na^+ is considered to be the sodium-dependent fraction of Gly transport. Parallel with the rise in the concentration of the Zn salt, the Na^+-dependent fraction of Gly enriching within the cell increased. Attention should be paid to the physiological fluctuation of the water content of the intestinal tissue. There was a significant correlation between the increase of the water content of the intestinal tissue and the quantity of Gly that entered from the mucosal solution or from the mucosal + serosal solutions. Possible mechanisms of action of the effects of pH and Zn are discussed.

Keywords. Glycine (Gly), transport, pig, jejunum, ^{3}H-Gly, ^{14}C-Gly, in vitro, zinc, pH.

During the last two decades numerous data have been obtained on the characteristics of glycine (Gly) transport. In their studies performed with isolated cells, Oxender and Christensen (1963) demonstrated the existence of four amino-acid transport systems (A, L, Ly^+, and ASC) and a specific Gly-transporting function in pigeon red blood cells. There are overlaps between the individual transporting systems. The influx of Gly into the cell is mediated mainly by system A and, to a lesser extent, by system ASC; correspondingly, Gly transport shows strong pH-dependence. System A renders possible strongly uphill processes; however, it is very sensitive to temperature, anoxia and metabolic inhibitors both in mesenchymal and epithelial cells, and is strongly Na-dependent (Christensen et al., 1952; Guidotti et al., 1978). Parallel with acidification of the medium, the role of system L in Gly transport gradually increases; however, the selectivity of the transport decreases (Guidotti et al., 1978).

According to the investigations of Guidotti et al. (1978) there is little difference between the transport systems of the different tissues. Other research workers treat the tissues with absorptive functions separately. In the rabbit ileum, at least two systems participate in the transport of neutral amino acids (Munck, 1981; Paterson et al., 1979). In the absence of sodium, Gly is absorbed less easily than the neutral amino acids of longer carbon chain (Smith, 1983).

Although numerous amino-acid absorption experiments have been conducted with nutritional purposes, the literature is poor in amino-acid transport studies of basic research nature, performed in pigs. Recently, Harmeyer et al. (1974) have studied in pigs the factors influencing the absorption of amino acids. Earlier, the present authors examined the effect of the Zn salt content of the feed on the absorption of Gly and other amino acids in Thiry-Vella-type isolated intestinal loop (Simon and Boldizsár, 1974; Boldizsár and Simon, 1980*a*, *b*). The increase of $ZnSO_4$ content was found to enhance the absorption of Gly and, to a lesser extent, aspartic acid (Asp), had no effect on the absorption of the other neutral amino acids studied, and strongly inhibited the absorption of arginine (Arg). In per os arrangement, the Zn salt added to the amino-acid mixture usually slowed down and prolonged the absorption of amino acids (Boldizsár and Simon, 1980*a*).

In the present work, the steps of the Gly absorption process were studied in vitro, in porcine small intestine, in the presence or absence of Na^+, at different pH values and Zn contents, using ^{14}C-Gly and ^{3}H-Gly.

Materials and methods

Experimental animals

Eight Dutch Landrace × Hungarian Large White pigs of 19 to 35 kg body mass were used. Before the experiment the pigs were fasted for 16 h; water was provided ad libitum.

Description of the operation

At the beginning of the experiment 25 mg/kg body mass Inactin and 28 mg/kg body mass Relaxil-G were given intravenously, in a self-dosing system, until anaesthesia was reached. The abdominal wall was opened; the blood vessels were ligated, and 1.5 m from the caecum in oral direction 6 cm long segments were excised from the middle part of the jejunum (five segments each on the right and the left). The mass of the intestinal segments was weighed. Between the segments, 1-cm-long portions were excised for determination of dry matter content. The intestinal segments were ligated half centimetre from the two ends, thus the average length of the intestinal sac was 5 cm. Ten intestinal segments were excised from each animal; thus, together with the 1-cm-long portions a 65-cm-long segment of the intestine was used. Excisions were performed at 3–4 min intervals. After the excision of intestinal segments the pigs were killed by bleeding.

In vitro method

The excised intestinal segment was washed in ice-cold saline, moisture was blotted up from its surface, the segment was bent to a loop, ligated at one end, suspended, and 2 ml Krebs-Ringer-HCO_3 (KR) solution of pH 7.4, was injected into it. Subsequently the other end of the sac was also ligated and the

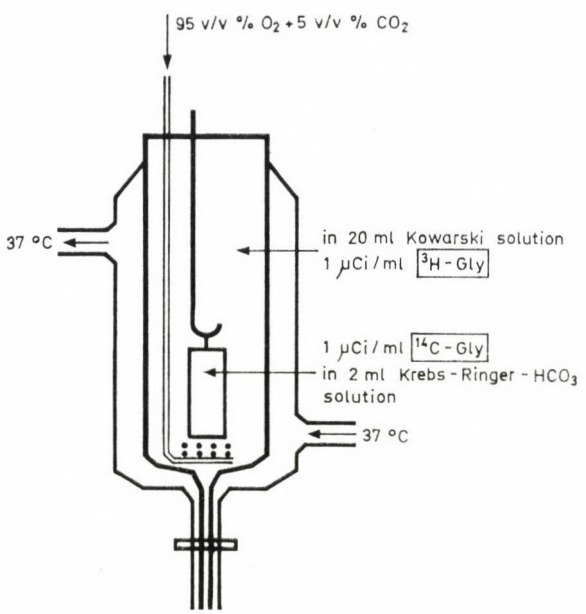

Fig. 1. Scheme of the in vitro system

sac was tied onto a looped glass rod and fixed in the incubation dish (Fig. 1). Eight to 9 min elapsed between the ligation of the intestinal segment and its placing into the medium. The preparation time needed to make a sac of the excised intestinal segment was 2 min. The medium outside the sac was a chloride solution according to Kowarski et al. (1974), the composition of which varied according to the purpose of the experiment. The experiment was carried out in a 20-ml, double-walled glass dish at 37 °C. A mixture of 95% O_2 and 5% CO_2 was perfused through the dish at a just countable bubble speed. After 60 min the sacs were removed from the dish in the same sequence as they had been placed into it, were washed thoroughly, moisture was blotted up from their surface, then the contents of the sacs were emptied into test tubes and their volumes were measured. The sacs were rinsed out with 2 ml KR solution and filled up to 5 ml. The mass of the empty sac was weighed, its two ends were cut off, and then the 5-cm middle portion was weighed again. An approximately 0.5 g part of this was hydrolysed during heating in 2 ml 40% KOH and reserved for radioactivity measurement. At the end of the experiment the activity of the dry matter content of the sac was determined.

Solutions

The serosal solution was a KR solution to which 18.5–25.9 kBq/ml ^{14}C-Gly and 15 mmol/l nonlabelled Gly were added.

On the mucosa side there were Kowarski's solutions, containing 29.6 to 59.2 kBq ^{3}H-Gly and 15 mmol/l nonlabelled Gly, and, in the sodium-free solutions 0.3 mol/l mannitol instead of NaCl. The pH of the mucosal solutions was adjusted to 7.4, 6.8 and 5.8. From all solutions, solutions of 0, 50 and 200 mg/kg Zn concentration were prepared by adding $ZnSO_4 \times 7\ H_2O$. Furthermore, Zn-free mucosal solutions containing 0.5 mmol/l DNP or 10 mmol/l NaIA, pH 6.8, were also made. The results were calculated to identical specific activities.

Radioisotope measurement technique

At the beginning and at the end of the experiment 0.1 ml of each solution was measured into 10 ml Packard Instagel scintillation solution; the mixture was shaken thoroughly, and the activities of the solutions were measured in a Packard Tricarb instrument in ^{3}H and ^{14}C channels. The background activity values of the cuvettes were determined previously. The efficiency of the measurement was determined, using standards of known extinction values; the activity of ^{14}C-Gly was measured also in the ^{3}H channel.

Mathematical analysis

A three-way analysis of variance (ANOVA) with two interdependent variables (pH and Zn) and one independent variable (Na^+) was performed, using the R_{40} computer of the Eötvös Loránd University of Arts and Sciences, Budapest, according to the BMDP2V programme of Dixon and Brown (1970).

In some cases Student's *t* test for one or two samples was used, and correlations and linear regression were calculated.

Results

The transport of Gly, in accordance with its function, was directed mostly towards the serosal solution. Although at the beginning of the experiment the quantity of nonlabelled Gly was the same (15 mmol/l) on both the mucosal and the serosal side, after 60 min 5–10 times as much labelled Gly had moved to the serosal side as to the mucosal side. Two to three times as much Gly entered the intestinal tissue from the mucosal side as from the serosal side. Thus, e.g. from a Na^+-containing, Zn-free solution of pH 7.4, about one-third of the Gly that entered from the mucosal side was transported to the serosal side (2504×10^3 dpm/g dry matter), while two-thirds (5153×10^3 dpm/g dry matter) were retained in the tissues. Only one-sixth (333×10^3 dpm/g dry matter) of the much less labelled Gly entering from the serosal side was transported to the mucosal side, and 5/6 remained in the tissues (Table IA and B).*

The amino acid content of the intestinal wall became considerably enriched within 60 min: in the presence of Na^+ up to 5.9–9.4 mmol/l tissue water in the different experimental groups (39–63% of the outside concentration); in the absence of Na^+ up to 4.3–5.5 mmol/l (29—37% of the outside concentration; Table II). In the case of a Na^+-containing, Zn-free mucosal solution of pH 7.4, 26% of the total Gly that entered the tissue (9649 dpm/g dry matter) was transported to the serosa side, only 3.4% towards the intestinal lumen, while more than 70% of it remained in the tissue. In the presence of Na^+, Zn resulted in a reduction of the Gly efflux from the tissue towards the serosal solution, which contributed to its enrichment in the intestinal tissue.

Gly transport showed a sodium-dependence in both directions. According to the expectations, this occurred, for the most part, during uphill processes, i.e. during Gly efflux from the tissue towards the mucosal solution or the serosal solution. The difference between values obtained in the presence and absence

*Naturally, the activity obtained for the intestinal tissue means not only the Gly taken up by the intestinal epithelial cells, but also the amino acids that had already been transported into the extracellular space; therefore, in a kinetic analysis, determination of the extracellular space of the intestinal tissue is essential (Smith, 1983).

Table IA

Sixty-min transport values of ^{3}H- and ^{14}C-labelled glycine (Gly) in vitro ($\bar{x} \pm SD\ 10^{-3}$ dpm/g dry matter)

A) NaCl-containing mucosal solution

pH	Zn-free		50 mg/kg Zn		200 mg/kg Zn	
	Mucosa → Cell Mucosa ← Cell	Cell → Serosa Cell ← Serosa	Mucosa → Cell Mucosa ← Cell	Cell → Serosa Cell ← Serosa	Mucosa → Cell Mucosa ← Cell	Cell → Serosa Cell ← Serosa
7.4	5153±1446 333±201	2504±1104 1659±1089	5948±2512 384±109	2499±897 2301±2310	4838±1097 432±273	1855±593 3231±1806
6.8	5834±554 327±189	1595±378 1848±1725	6576±2147 339±207	1313±2022 2331±2022	7073±2465 267±144	1093±634 2745±2157
5.8	5793±746 177±81	1141±1144 1722±1089	4972±882 108±78	727±483 1356±948	4919±897 126±87	1021±1091 1221±831
6.8	DNP (0 Zn)	3900±696 111±99	801±888 1776±1308	NaIA (0 Zn)	2932±563 51±36	415±287 1506±1284

DNP = 2, 4-dinitrophenol; NaIA = sodium iodoacetate.

Table IB

Sixty-min transport values of ^{3}H-and ^{14}C-labelled glycine (Gly) in vitro ($\bar{x} \pm SD\ 10^{-3}$ dpm/g dry matter)

B) Mannitol-containing mucosal solution

pH	Zn-free		50 mg/kg Zn		200 mg/kg Zn	
	Mucosa → Cell Mucosa ← Cell	Cell → Serosa Cell ← Serosa	Mucosa → Cell Mucosa ← Cell	Cell → Serosa Cell ← Serosa	Mucosa → Cell Mucosa ← Cell	Cell → Serosa Cell ← Serosa
7.4	4342±1088 72±15	568±298 1242±699	3835±1112 96±48	571±366 1173±846	3618±402 60±30	584±364 1263±948
6.8	4564±2020 81±27	861±593 1155±585	3410±435 84±30	842±693 810±525	3878±508 63±27	493±357 1146±576
5.8	4098±823 72±51	450±62 1425±639	3581±1160 63±18	474±291 1443±891	3794±474 75±15	431±489 1248±921

of Na^{+} can be considered the Na^{+}-dependent fraction of Gly transport. Comparing the results obtained with the NaCl-containing and the mannitol-containing mucosal solutions it appears that in the absence of Na^{+} the efflux from the tissue into the mucosal solution decreased by 40–86% in the different groups ($P < 0.05$): thus, for the Zn-free mucosal solution of pH 7.4 it decreased from 333 to 72×10^{3} dpm/g, i.e. by 79%; the efflux towards the serosal solution

Table II

Accumulation of glycine (Gly) in the intestinal tissue
(μmol Gly/ml tissue water)

Mucosal solution		Na-containing			Mannitol-containing		
Zn content, mg/kg		0	50	200	0	50	200
pH	7.4	6.55 ±2.13	7.97 ±4.37	7.79 ±1.82	5.39 ±1.42	4.84 ±1.88	4.71 ±1.26
	6.8	7.42 ±1.15 *5.48 ±1.62 **4.29 ±1.71	8.61 ±3.37	9.02 ±3.65	5.52 ±1.85	4.08 ±0.87	4.85 ±0.92
	5.8	7.26 ±1.19	6.12 ±1.52	5.95 ±1.05	5.33 ±0.99	4.85 ±1.16	4.87 ±0.99

* DNP-containing solution; ** NaIA-containing solution.

decreased by 35–77% ($P < 0.05$). In downhill processes the effect of Na^+ was less pronounced, but still present: the influx of Gly from the mucosal solution into the tissue decreased by 16–48% ($P < 0.02$). With the exception of one group, the mean values obtained for Gly transport from the serosal solution towards the tissue were also lower in the absence of Na^+ (maximum by 65%); however, according to the ANOVA, the effect was not significant in this respect (Figs 2a and 2b).

According to the ANOVA, the effects of Na^+ and pH were in significant interaction ($P < 0.01$), expressed partly in the fact that the effect of Na^+ on the transport decreased at a pH shift to the strong acid direction (pH 5.8), and partly in the fact that pH had an effect on transport only in the presence of Na^+. The pH shift in the acid direction significantly reduced uphill amino-acid efflux from the tissue towards both the mucosal ($P < 0.01$) and the serosal ($P < 0.01$) solutions.

In the presence of Na^+ the Zn salt reduced Gly efflux from the tissue into the serosal solution ($P < 0.05$).

The enrichment of labelled Gly in the tissue tended to depend on the presence of Na^+ in the mucosal solution ($P = 0.064$).

In the intestine, passage across the basolateral membrane from the tissue into the serosal solution seems to be the limiting step of amino-acid absorption. In the most favourable case, one-third of the Gly taken up by the tissue from the mucosal solution had been transported into the serosal solution by the end of the 1-h period of experiment.

The experiment conducted with DNP and those with mannitol yielded results of the same tendency: the specific inhibitor (DNP) reduced Gly absorp-

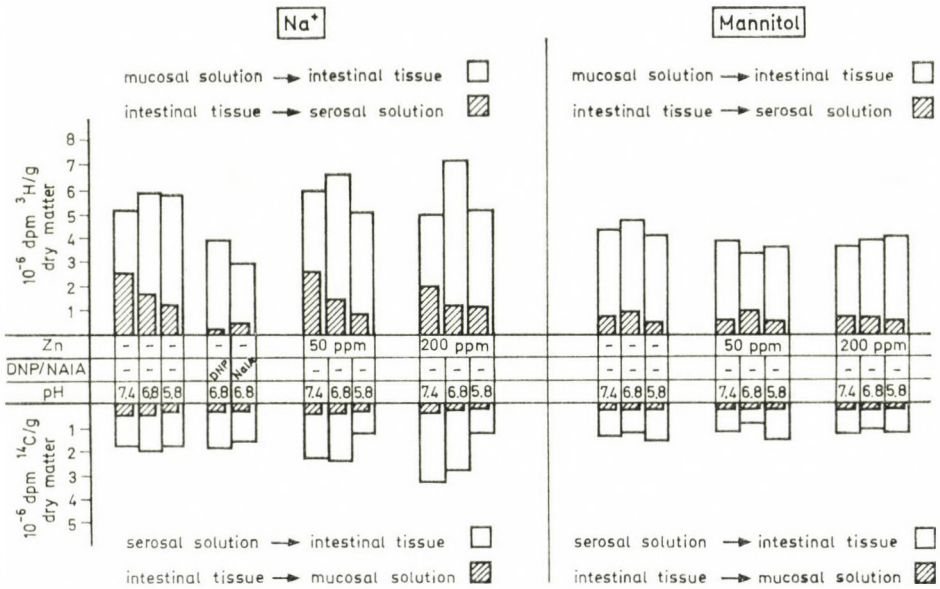

Fig. 2. Comparison of the partial processes of the bidirectional transport in the different experimental designs. *A)* Na-containing mucosal solution; *B)* Mannitol-containing mucosal solution. (DNP = 2,4-dinitrophenol; NaIA = sodium iodoacetate)

tion to the level obtained with mannitol (in the mucosal solution-to-tissue direction this decrease is significant ($P < 0.05$) as compared to the Na^+-containing solution). NaIA caused a further decrease in the transport steps playing a role in absorption. Only one-fourth of the activity obtained in the presence of Na^+ moved from the mucosal into the serosal solution (in the mucosal solution → tissue direction: $P < 0.01$; in the tissue → serosal solution direction: $P < 0.02$).

The Na^+-dependent fraction of the Gly accumulating in the tissue increased with the rise of Zn concentration of the mucosal solution.

As regards the osmotic conditions of the media and the changes in the dry matter and water content of the tissues, the following facts can be established: the osmolarity of the Kowarski's solutions (330 mosmol, and 331.5 and 336.0 mosmol in Zn-containing solutions) and of the KR-HCO_3 (327.7 mosmol) was identical with the hypothetical osmotic conditions of tissue cells. The mannitol-containing solution (350, and 351.5 and 356.0 mosmol) was slightly hyperosmotic as compared to the tissue.

During the 1-h period of incubation, the intestinal tissue took up water from the incubation media, thus the relative dry matter content of the tissue decreased. E.g., in the group where a Na^+-containing, Zn-free mucosal solution of pH 7.4 was used, the dry matter content decreased from 18.08 to 14.24%; this means that 100 g tissue took up a further 26.96 g water, which is 33.6% of the original 81.92 g water content (thus, the percentile water content of the

tissue rose to 85.76%). In experiments with Na-containing mucosal solutions the increase of water content, as related to the initial water content, was between 20.9 and 48.2% in the different groups; the change was statistically significant in all groups but one (see Table IV). In the groups where mannitol was used, water uptake was of a lesser degree (between 0.7 and 14.0%); here, with the exception of two groups, the increase was not significantly different from 0, and neither was in the DNP and NaIA groups.

The majority of the fluid entered the tissue from the mucosa. Namely, the volume of the 2 ml serosal fluid decreased maximum by 20% (as calculated on the basis of re-measurements and the radioactivity of the washing fluid), which can account only for a small fraction of the increase in the water content of the intestinal tissue.

According to the ANOVA, the influx of water into the tissue depends on the presence of Na^+ in the mucosal solution ($P < 0.001$); i.e. the influx of water is higher in the presence of Na^+, and also on pH ($P < 0.01$), since in the presence of Na^+, parallel with the pH shift of the mucosal solution in the acid direction the influx of water into the intestinal tissue decreases. The influx of Gly from the mucosal solution into the intestinal tissue (^{3}H-Gly) was in correlation with the percentile water uptake ($r = 0.536$; $n = 80$; $P < 0.001$); the regression is linear: $y = 5.60 \times 10^{-3}x - 7.30$, where $x = {}^3$H-Gly cpm/g dry matter. The overall quantity of Gly entering from the mucosal and serosal solutions ($^3H + {}^{14}$C-Gly) also correlated with the percentile water uptake ($r = 0.459$; $n = 80$; $P < 0.001$; linear regression: $y = 3.19 \times 10^{-3}x - 1.26$, where $x = {}^3$H-Gly $+ {}^{14}$C-Gly cpm/g dry matter). The correlation was significant even if examined for groups broken down according to the pH.

The dry matter content of the control tissue was determined in 4–10 segments of the small intestine (jejunum) taken from each of 8 pigs. The deviation between different intestinal segments of a given animal and the methodological deviation were low (the mean coefficient of variation was 4.5%); on the other

Table III

Dry matter content of the intestinal (jejunal) tissue before the experiment

Serial number of experimental animals	Number of samples (n)	Dry matter/g wet tissue ($\bar{x} \pm$ SD)
1	5	15.99 ± 0.53
2	4	18.83 ± 1.07
3	4	18.85 ± 1.23
4	5	16.57 ± 0.64
5	9	19.53 ± 1.34
6	9	19.47 ± 0.47
7	10	19.50 ± 0.36
8	10	20.56 ± 0.68
Total: 8	56	19.00 ± 1.59

Table IV

Water uptake of intestinal tissue as compared to its water content measured before the experiment, in %

	Na^+-containing mucosal solution			Mannitol-containing mucosal solution		
	0	50	200	0	50	200
	mg/kg Zn			mg/kg Zn		
pH 7.4	33.6 ± 6.8 $P < 0.01$	48.2 ± 10.3 $P < 0.01$	21.7 ± 19.2 N. S.	14.0 ± 8.0 $P < 0.05$	9.7 ± 4.0 $P < 0.02$	4.4 ± 7.8 N. S.
pH 6.8	30.3 ± 13.5 $P < 0.05$	38.8 ± 10.6 $P < 0.01$	35.2 ± 22.1 $P < 0.05$	15.3 ± 11.4 N. S.	12.4 ± 9.3 N. S.	5.2 ± 4.4 N. S.
pH 5.8	27.8 ± 4.8 $P < 0.02$	29.8 ± 10.3 $P < 0.02$	20.9 ± 3.5 $P < 0.01$	5.5 ± 8.9 N. S.	0.7 ± 8.0 N. S.	3.0 ± 7.8 N. S.

DNP: 12.3 ± 11.4; N. S.
NaIA: 6.6 ± 9.4; N. S.
N. S. = not significant.

hand, there were significant differences between the animals (even the least difference, i.e. that between animals no. 7 and 8, was significant ($P < 0.001$; Table III).

Discussion

The considerable individual variation observed at the beginning of the experiment in the dry matter content of the intestinal tissue (Table III) can be attributed, for the most part, to the different water uptake due to the ad libitum water supply. Harmeyer et al. (1974) and Matthews and Laster (1965) emphasized the considerable variations existing in the transport rate between animals in experiments on intestinal amino-acid transport. The latter authors suggested that water might have a role in the transportation of amino acids accumulated in the cell. Therefore, the physiological water content of the tissues should be considered in absorption experiments.

In studies of transport, the question of the movement of water between the medium (media) and tissue cells always arises. During the 60-min incubation a considerable water influx from the mucosa side was observed, primarily when using a Na^+-containing Kowarski's solution which has nearly identical osmolarity with the tissues. On the other hand, water inflow into the cell from the serosal solution of similar osmolarity was negligible as well as that from the slightly hypertonic, mannitol-containing mucosal solution. Water influx into the cell from the DNP-containing solution which had the same osmolarity as the Na-containing mucosal solution was also of low degree. Although in the experiments of Christensen et al. (1967) the influx of amino acids was not accompanied by a major alteration in the intracellular water content, organic

factors, amino acids and sugars are known to promote the transport of water and Na (Hellier et al., 1973). Although water is transported passively, its transport is connected with the movement of the total solute (e.g. sugar). According to Barry et al. (1961), the water has two fractions: a glucose-dependent and a glucose-independent. Our data show that the amino-acid transport was accompanied by water flux, and there was a significant correlation between the water and Gly content of the intestinal tissue. In the experiments of Matthews and Laster (1965) the transport of 0.8 mmol/g dry matter/h Gly was followed by transport of 8.8 ml/g dry matter/h water; thus the water movement observed by us cannot be considered unreal.

As regards the high Gly content of the media, our experimental design markedly differed from the usual. The amino-acid concentration of the bathing fluids is usually a few mmol/l (Christensen, 1979; Al-Saleh and Wheeler, 1982; and others). The Gly transport consists of two components: at a lower concentration of the outside medium an active, saturable component, and at a higher concentration (in the experiments of Revsin and Morrow (1976) above 10 mmol) a non-saturable component. The latter is transported through a simple diffusion process (Oxender and Christensen, 1963). The rise of the outside concentration increases the uptake of most amino acids. The sodium-independent flux increases in linear proportion to the extracellular amino-acid concentration, whereas the sodium-dependent flux increases in a non-linear manner and is saturable. At a Gly concentration of 10 mmol, the relative influx is only 18% (Al-Saleh and Wheeler, 1982). At such concentrations the saturable processes constitute only a small part of the whole; the larger part is made up by non-saturable and sodium- and pH-independent processes. The present studies yielded similar results: when the Gly concentration of the mucosal solution was 15 mmol, 52 to 84% of the transport from the mucosal solution towards the tissue was sodium-independent. A kinetic study could determine the characteristics of saturation in the mucosal cell under the conditions of our experimental design.

Understandably, the need to differentiate between active and passive processes has compelled researchers of transport processes to work with low amino-acid concentrations. In the present experiments we attempted to simulate the practical conditions by using high amino-acid contents, longer absorption period, an acidic or slightly basic medium on the mucosal side and a slightly basic one on the serosal side (taking into account that the pH values of the bile, cell, jejunum and blood are 5.3–7.1, 6.8, 7.4–8.7 and 7.4, respectively). For the same reason and in accordance with the purpose of the experiment, NaCl was omitted from the mucosal solution, while the serosal solution always contained NaCl. The same reason justified the use of an optimum Zn concentration (50 ppm; Mészáros, 1973) and supra- and suboptimal Zn concentrations. In acid media, Kowarski's solution containing chloride as anion was used to make Zn

salts soluble. It should be mentioned that the addition of 15 mmol/l Gly to the solutions eliminated the turbidity of the solutions of pH 6.8 and 5.8, obviously due to the formation of a Zn-glycinate complex.

In the present study, maximum one-third of the activity taken up by the tissue from the mucosal solution got into the serosal solution and two-thirds were retained in the tissue. In the experimental design of Harmeyer et al. (1974), 80% of the activity taken up from the mucosa remained in the intestinal tissue. It is known that the initial influx of Gly does not indicate in advance the degree of steady state accumulation. The influx of Gly is slow and is in linear relationship with time until reaching a distribution of 1 : 5; its efflux is even slower, indicating that the entry of Gly into the cell and its exit from there are mediated by different mechanisms. Probably, the amino acids use the transport systems in a different degree during their entry into the cell and during their exit from there. Due to the slow efflux of Gly it can become enriched in the cell. The efflux of Gly cannot be a simple diffusion since amino acids of longer carbon chains exit more rapidly, and in a simple diffusion the transport speed is the same in both directions (Oxender and Christensen, 1963).

Of the free amino acids Gly is present in the cell in the highest concentration; the other amino acids obtain the energy required for their transport through the transport of Gly (Eavenson and Christensen, 1967). Although Gly takes part in numerous metabolic reactions, concentration changes due to its metabolism which could influence the transport need not be reckoned with at the applied concentrations of media.

By comparing the kinetic constants of transport across the basolateral membrane and across the brush-border, it was found for some amino acids that the limiting step of transport rate was associated with the basolateral membrane (Munck, 1981). Also in the experimental design applied by us, passage towards the serosal solution has proved to be the limiting component of absorption. A sodium-independent, equilibrating transport mechanism and, for alanine, three sodium-dependent mechanisms are hypothesized to exist in the passage across the basolateral membrane (Munck, 1981). Its regulation is independent of that of the luminal membrane. According to Oxender and Christensen (1963) a part of the Gly efflux is pH-sensitive (similarly to the influx mediated by transport system A), whereas another part is mediated by system L. The increased tissue-to-serosa efflux means mostly exchange processes and not a net efflux. Although in our experiment also the serosal solution contained Gly in a high concentration, the serosa-to-tissue passage was of low degree, which was in agreement with results of the studies mentioned above.

The pH, Na^+- and Zn^{2+}-content determining the degree of transport and the electrical potential difference (PD) measurable in the intestine are in a close relationship. The PD value measured in vivo in rat jejunum and ileum was 7.9 and 4.4 mV, respectively, while in vitro 3.9 and 4.4 mV (Hajjar et al., 1969);

in everted small intestinal sacs of pigs Harmeyer et al. (1974) measured a PD of 3.9 mV. The mucosal side is negative and the serosal side positive. The PD depends on the presence of actively-transported sugars on the mucosal side (Lyon and Crane, 1966) and on the Na^+ content of the medium. In the presence of glucose the short-circuit current is equal to the sodium transfer of the luminal medium (Clarkson and Toole, 1964). Presumably, amino acids can also play the above role of sugars, at least in part.

According to Kowarski et al. (1974), in everted intestinal sac the transport of Zn is an active, Na^+-dependent process, which takes place also against the electrochemical potential gradient. The entry of Zn into the intestinal epithelial cell is rapid. As compared to this, it proceeds slowly towards the serosa (Sahagian, 1967). In aqueous solution Zn forms chelates with the amino acids which increase the solubility of Zn salts in acid solutions. In our earlier in vivo studies a Zn salt injected into an isolated intestinal loop promoted the disappearance of Gly from the intestinal lumen, and the Zn salt prolonged the absorption of an amino acid mixture given per os; all this was true for the kinetics of Zn as well (Simon, 1978). The processes mentioned above were presumably due to metal complex formation. It has long been known that the absorption of amino acids comes to a standstill if the -COOH or the $-NH_2$ radical changes (Wilson, 1962). Based upon our present in vitro studies an increased influx from the mucosa cannot be proved, the passage into the serosal solution decreased, and simultaneously the accumulation of the Na^+-dependent Gly fraction in the intestinal wall increased.

With rising $ZnSO_4$ concentrations the mucosal solution becomes more and more acidic (the pH of a 50 mg/kg Zn solution is 6.8, while that of a 200 mg/kg Zn solution is 5.8). Our results have shown that a shift in the pH of the mucosal solution in the acid direction decreases the transport from the tissue into the serosal solution.

Transport demands a carboxyl group and an α-amino or imino group, and, in the case of neutral amino acids, an uncharged side chain (Munck, 1981). The positive or negative charge of the side chain determines the mode of transport, i.e. that it takes place by the mediation of a neutral, cationic or anionic amino acid transport system. With the alteration of pH, amino-acid uptake changes according to the charge of the side chain (Christensen, 1979).

In the Ehrlich ascites tumour cell the transport system of Gly is sodium-dependent. In pigeon red blood cells the transport of Gly decreases with the drop of pH; at pH 5.0 it is only 30% of the value found at pH 7.4 (Eavenson and Christensen, 1967).

In our experiments the effect of Na^+ on transport was found to decrease with acidification. This can be explained by the observation of Csáky (1969) according to which even a small decrease of the pH is sufficient to inhibit the sodium pump.

Cytotoxic substances strongly inhibit the accumulation of Gly: DNP, Cu salt and NaIA resulted in an inhibition of 50, 65 and 75%, respectively (Revsin and Morrow, 1976). In Na-free solutions DNP causes a further reduction of influx in chicken intestine, indicating that sources other than the Na-gradient are also used by the cell as energy sources of amino-acid metabolism (Munck, 1981). Transport is known to require also an SH group (Revsin and Morrow, 1976). NaIA inhibits the energy-producing processes obviously by blocking the SH-containing glyceraldehyde-3-phosphate dehydrogenase enzyme of glycolysis. NaIA causes a more pronounced reduction in transport than DNP (Eavenson and Christensen, 1967) which, according to our present results, holds true also for the porcine intestine. In a 150-min study of everted intestinal sac, Harmeyer et al. (1974) found an approximately 25% reduction in the uptake and output of amino acids due to the effect of DNP. In our experiments the effect of DNP was of a similar magnitude. Gly uptake dropped to the level found in the absence of sodium. NaIA resulted in a further decrease of Gly transport.

When interpreting the alterations in Gly transport that are due to changes of pH, Na^+- and Zn-content and PD, the following processes should be considered: (1) The properties (selectivity, affinity) of the transport systems used by Gly undergo changes; (2) the configuration of Gly changes (protonation, binding of Zn to the amino acids in different forms, competition between $H^+/Na^+/Zn^{2+}$); (3) the proportion of transport systems used by Gly is modified; (4) due to ionic alterations the potential difference (PD) between the two sides of the intestinal tissue suffers a change.

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EFFECT OF BETA-CAROTENE SUPPLEMENTATION ON THE POSTPARTUM RESUMPTION OF OVARIAN CYCLICITY IN COWS KEPT ON CAROTENE-DEFICIENT DIET

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The effects of carotene deficiency and additional beta-carotene supplementation on the postpartum resumption of ovarian cyclicity were investigated. From the 20th day before calving up to postpartum days 62–70, 400 mg/animal/day synthetic beta-carotene (Rovimix-β-carotene 10%, Hoffmann–La Roche, Basel) was mixed in the fodder of 12 cows *(treated group)* of a stock that had been kept on a carotene-deficient diet for a long time. Further 11 cows served as *control.* Blood samples were collected from each animal on 4 occasions (about 20 days before parturition and on days 1–5, 30–35 and 60–65 post partum), and their beta-carotene, non-esterified fatty acid (NEFA) and total cholesterol (TCh) concentrations were assayed. The postpartum resumption of cyclic ovarian activity was monitored by regular clinical examinations and by determination, by radioimmunoassay (RIA), of the progesterone profiles of milk samples collected twice weekly up to days 70–75 post partum. The animals with increased postpartum lipid mobilization (NEFA concentration, days 1–5: > 0.80 mmol/l, days 30–35: > 0.40 mmol/l) were excluded from the evaluation.

Data for 10 treated and 11 control cows were evaluated. By day 70 post partum, the ovaries of all cows had resumed cyclic activity. In the treated group the luteal phase started, on the average, about 10 days earlier than in the control group; however, the difference was not significant. The treated cows were usually characterized by a low incidence of large ovarian cysts and relatively higher progesterone concentrations during their luteal phases.

As shown by linear regression, correlations existed between the time of resumption of ovarian activity and the NEFA level, and also between the integrate of progesterone concentrations and the NEFA and beta-carotene levels.

The results have confirmed that carotene supply has an important, but as compared to energy supply probably only a secondary, effect on the postpartum resumption of ovarian cyclicity.

Keywords. Beta-carotene, ovarian cyclicity, post partum, carotene deficiency, cow.

The physiologically possible soonest postpartum conception of cows is one of the decisive factors in an economical function of cattle breeding. In the biological sense, the length of the service period is determined by the rate of involution and by the postpartum resumption of cyclic ovarian activity.

On the ovaries of healthy, non-suckling cows already a few days after parturition there are follicles exceeding 10 mm in size, which are soon followed by the appearance of the first corpus luteum (CL) and a simultaneous rise of

serum and milk progesterone (Morrow et al., 1969; Lamming and Bulman, 1976; Karg and Schallenberger, 1983).

The postpartum resumption of cyclic ovarian activity is influenced, besides numerous other factors, by feeding. First of all the importance of a satisfactory energy supply should be emphasized (Morrow, 1970; Peters and Riley, 1982; Haraszti et al., in press), but a similar role of certain deficiencies (macro- and microelements, vitamins) may also be hypothesized.

The reproductive-biological significance of vitamin A and carotene supply is well-known. The existence of a specific, vitamin A-independent, physiological effect of beta-carotene on the ovarian activity of the cow already seems to be proved (Meyer et al., 1975; Lotthammer et al., 1976; Lotthammer and Ahlswede, 1977; Schams et al., 1977; Ahlswede and Lotthammer, 1978). In the present work the influence of carotene deficiency and of beta-carotene supplementation of carotene-deficient cattle on the postpartum resumption of cyclic ovarian activity was investigated under large-scale management conditions.

Materials and methods

For the experiment, a dairy farm was chosen where the serum beta-carotene level of the dry cows (a value reflecting their supply status) had been found very low (50–100 μg/dl) for several months.

In agreement with the practice common in Hungary, feeding was based on corn silage completed with poor-quality alfalfa hay, fodder and mineral premix (Table I). Feeding of green fodder (about 15 kg/animal/day mixture of freshly cut wheat and vetches) was started before the end of the experiment, 55–65 days after the cows had calved. Simultaneously, the daily dose of corn silage was reduced to 15 kg; the quantity of alfalfa hay, fodder and mineral premix remained unchanged.

In February, 23 healthy, 3–10 years old Holstein-Friesian $\times$ Hungarian Fleckvieh F_1 cows of better than average body condition and being in their 2nd to 7th pregnancy were selected for the experiment. Their calving was expected to occur in the second half of March. The animals were randomly allotted to two groups matched for age and milk production (Table II). To the fodder ration of 12 cows (treated group) a daily dose of 400 mg synthetic beta-carotene (Rovimix-β-carotene 10%, Hoffmann–La Roche, Basel) was added from about the 20th day before parturition up to days 62–70 post partum. The remaining 11 cows received no carotene supplementation to serve as control.

The animals were kept under strict observation. After parturition, the status of the genital organs was checked by clinical (rectal, vaginal) examination at 10- to 14-day intervals. Oestrous cows were inseminated, but no hormo-

Table I

Feeding of the experimental animals up to the beginning of green feeding

	Dry cow		Lactating cow
	Weeks 1–6	Weeks 7–8	
Alfalfa hay, poor[1]	4 kg	5 kg	4 kg
Corn silage, medium quality	20 kg	10 kg	25 kg
Dairy cow diet[2]	1 kg	5 kg	0.4 kg/l milk[3]
Malt sprout	—	—	1 kg
Bran	1 kg	1 kg	—
Neonatrophor premix	0.25 kg	0.25 kg	0.15 kg

Remarks: [1] Soaked in swatch, heavily contaminated with weeds.
[2] Composition: 30% barley groats, 68% corn-grits, 1% AP-18 premix, 0.5% cow premix no. 21, and 0.5% fodder salt.
[3] The dairy cow diet was given only for the part by which daily milk production exceeded 10 l.

Table II

Parameters of the treated (beta-carotene-supplemented) and control groups ($\bar{x} \pm SD$)

	Treated (n = 12)	Control (n = 11)
Age, years	5.6 ± 2.0	4.8 ± 1.6
Number of calvings	3.7 ± 1.6	2.9 ± 1.1
Milk production, l		
in the previous 300-day lactation	5300 ± 917	5096 ± 767
between days 30 and 60 of the lactation under study	27.7 ± 4.3	27.1 ± 4.9

nal treatments which could have influenced ovarian activity were performed. The observed pathological alterations and necessary treatments were recorded as well as the times of oestruses, inseminations and conceptions. Data collection was continued up to day 150 post partum.

On four occasions, about 20 days before parturition (i.e. in the treated group before the start of carotene supplementation), within 5 days after parturition, and between days 30–35 and 60–65 post partum, blood samples were taken from the cows. The beta-carotene concentration of the serum was determined as described by Brubacher and Vuilleumier (1974) and modified by Somorjai and Pethes (1984). The non-esterified fatty acid (NEFA) concentration of the serum was measured by Duncombe's (1964) method, and its total cholesterol (TCh) concentration according to Rappaport and Eichhorn (1960).

From parturition up to days 70–75 post partum milk samples were taken twice weekly from each cow at the end of the morning milking for progesterone determination. The samples were preserved in 0.1% sodium azide, defatted by centrifugation and stored at +4 °C until analysed. The progesterone concentra-

tion of defatted milk samples was assayed by RIA, without extraction as described by Oltner and Edqvist (1981) and modified by us (Pethes et al., 1984; Pethes and Kulcsár, 1984). The RP 7/7 antibody produced by Csernus (1981) was used.

Based upon previous experience (Haraszti et al., 1982; Haraszti et al., in press), data of animals showing signs indicative of increased lipid mobilization (NEFA concentration within 5 days after parturition: >0.80 mmol/l; between days 30–35: >0.40 mmol/l) were excluded from the evaluation.

From the results, individual progesterone profile curves were drawn for each animal up to days 70–75 post partum. Considering a progesterone level exceeding 1.6 nmol/l to be indicative of luteal function, as proposed by Oltner and Edqvist (1981), the number of commenced CL phases, the length of completed CL phases and the maximum progesterone concentration measured during the CL phases for the period up to postpartum day 70 were determined for each animal. Averages were calculated for each group. Subsequently, the individual progesterone concentrations measured up to day 70 were integrated by the method of Haraszti et al. (in press), taking into account the time of sampling. Thus, the sum of all progesterone concentrations measured until then for the given animal was characterized by the area under the integral curve determined for the time under study, well reflecting the resumption of ovarian functioning. This method permits us to characterize the cyclic postpartum progesterone production by a single index suitable for use in any statistical procedure.

Statistical evaluation of the data was performed by Student's *t* test and a four-square table. Correlations between parameters were examined by linear regression.

Results

The data of two treated cows with increased lipid mobilization were excluded from the evaluation. Both of these cows rapidly lost bodyweight after parturition: in 5–6 weeks' time they lost about 20% of their body mass. Their progesterone profiles indicated that neither had resumed cyclic ovarian activity by postpartum day 70.

As a result of carotene feeding, the beta-carotene level of the treated cows slightly rose up to the time of calving, then it became stable at a level around 100 μg/dl. In control cows, the carotene concentration dropped to the half or one-third of the initial value at calving, then it tended to rise slowly. The difference between the two groups was significant at calving and between postpartum days 30 and 35. The serum beta-carotene levels are shown in Fig. 1.

In the peripartal period the changes of the NEFA and cholesterol levels showed no inter-group difference and corresponded to the physiological con-

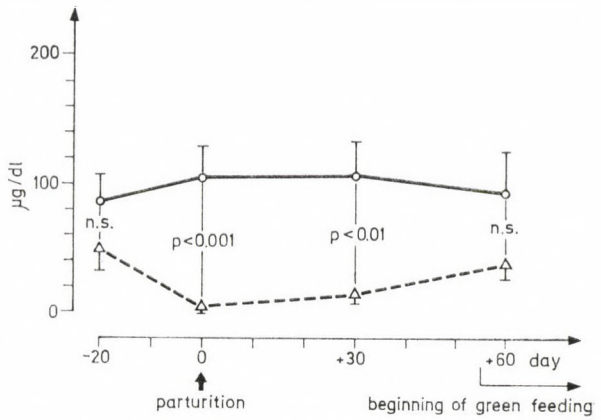

Fig. 1. Blood serum beta-carotene concentration (○ — ○ beta-carotene (400 mg/day); △ - - - △ control)

ditions. In the first two postpartum samplings NEFA concentrations close to the upper limit of the physiological range, although not pathological, were measured frequently (days 0–5: 0.60–0.75 mmol/l; days 30–35: 0.30–0.35 mmol/l).

No retention of placenta occurred. Animals receiving carotene supplementation showed a more rapid involution, which, in the morphological sense, had been completed by days 28–35 as shown by rectal palpation. In control cows, involution was completed about 10–14 days later. No important difference was found between the treated and the control group in the number of uterine treatments needed during the first 30 days of the postpartum period. In the treated group only one cow required uterine treatment after postpartum day 30, whereas in the control group six animals had to be treated.

After calving, in the period of serial progesterone measurements, all of the 11 control and 10 treated cows showing no signs suggestive of pathological lipid mobilization resumed cyclic ovarian activity. Usually 1 to 3 CL phases per animal passed off up to postpartum day 70; in one of the control cows and in one treated cow 4 CL phases passed off or commenced during the above period. In the period of observation altogether 51 cycles (Table III) were followed up in the two groups. Data characterizing the first, second and third postpartum CL phases are presented in Table IV. The 4th CL phase started on day 70 in both of the observed cases. The most characteristic ovarian abnormity was the delayed (after day 28) resumption of cyclic ovarian activity (Table V). Of the metabolic parameters determined, only the NEFA concentration showed a statistically significant correlation with the delayed resumption of ovarian cyclicity (Table VI). Although the treated cows started cycling about 10 days earlier on the average, due to the large individual variation the difference was not significant. The number of the other ovarian disorders observed, except the

Table III

Distribution of cycles examined clinically and by progesterone analysis

	Treated[1] (n = 10)	Control (n = 11)
Total number of cycles examined	27	24
Of this, completed cycles	18	14
cycles started but not completed until day 70	6	8
conception	3	2
Average number of cycles, $\bar{x} \pm SD$	2.2 ± 0.9	2.7 ± 0.6

Remark: [1] Data of the two cows showing pathological lipid mobilization were excluded from the evaluation.

Table IV

Characteristics of the first, second and third postpartum CL cycles

		First CL phase			Second CL phase			Third CL phase		
		Beginning[2]	Duration[3]	Maximum progesterone concentration[4]	Beginning[2]	Duration[3]	Max. prog. conc.[4]	Beginning[2]	Duration[3]	Max. prog. conc.[4]
		day		nmol/l	day		nmol/l	day		nmol/l
Control (n = 11)	$\bar{x}$	38.0	8.5	7.31	46.9	12.0	10.48	56.2	15.5	9.46
	±SD	18.7	4.0	6.12	16.0	2.7	9.91	8.2	3.5	0.85
	n	11	8	11	8	4	4	4	2	3
Treated[1] (n = 10)	$\bar{x}$	27.9	8.7	10.12	44.6	9.0	11.63	58.8	13.0	19.68
	±SD	7.5	3.8	10.53	10.2	3.5	8.32	8.7	—	10.88
	n	10	10	10	10	7	10	6	1	4

Remarks: [1] Without the data of the two animals showing pathological lipid mobilization.
[2] Based upon the data of all cows that had begun the given CL phase.
[3] Based upon the data of all cows that had completed the given CL phase.
[4] Based upon the data of all cows that had completed the given CL phase or had got beyond the 12th day of the given CL phase.

Table V

Times of beginning of the first CL phase indicative of the postpartum resumption of ovarian cyclicity

Days after parturition	Treated[1] (n = 10)	Control (n = 11)
≤28	6	5
29–35	3	
36–42		
43–49	1	1
50–56		3
57–63		1
64–50		1

Remark: [1] Without the data of the two animals showing pathological lipid mobilization.

Table VI

Correlations of certain metabolic parameters with the beginning of the first postpartum CL phases indicative of the resumption of cyclic ovarian activity

Characteristics of the regression line[1]		Treated[2] (n = 10)	Control (n = 11)	Total (n = 23)
Days 1–5 post partum				
NEFA	A	0.303	0.428	0.382
	B	0.010	0.003	0.006
	r	0.447*	0.310	0.512*
Total cholesterol Beta-carotene		No correlation can be established		
Days 30–35 post partum				
NEFA	A		0.231	0.225
	B		0.002	0.003
	r		0.397*	0.393*
Total cholesterol Beta-carotene		No correlation can be established		

Remarks:

[1] The characteristics of the regression line are given only for $r > 0.300$ and t_B: min. $P < 0.05$.

[2] Without the data of the two cows showing pathological lipid mobilization.

* r: significant (min. $P < 0.05$).

Correlations were not found with any of the parameters determined in samples taken around day 20 before calving and between days 60–65 post partum.

Table VII

Incidence of ovarian disorders

	Treated (n = 10)	Control (n = 11)
(1) *Disturbances of the CL phase* (progesterone >1.6 nmol/l)		
Short CL phase[2]	10	6
Of this, 1st postpartum CL phase	6	5
Corpus luteum persistens[3]	1	—
(2) *Ovarian cysts*[4]		
Follicle-theca cyst[5]	1	3
Follicle-lutein cyst[6]	—	2
(3) *Other abnormities*		
Cessation of cycling[7]	2	1

Remarks:

[1] Without the data of the two cows showing pathological lipid mobilization.

[2] Duration: ≤10 days.

[3] Duration: ≥20 days, in previously non-inseminated animals.

[4] Formations of about ≥20 mm in size, palpable for at least 15 days on the surface of the ovary.

[5] Progesterone level <1.60 nmol/l.

[6] Progesterone level ≥1.61 nmol/l.

[7] Progesterone concentration <1.60 nmol/l for at least 15 days, without the simultaneous presence of cysts in animals which had already commenced cycling once.

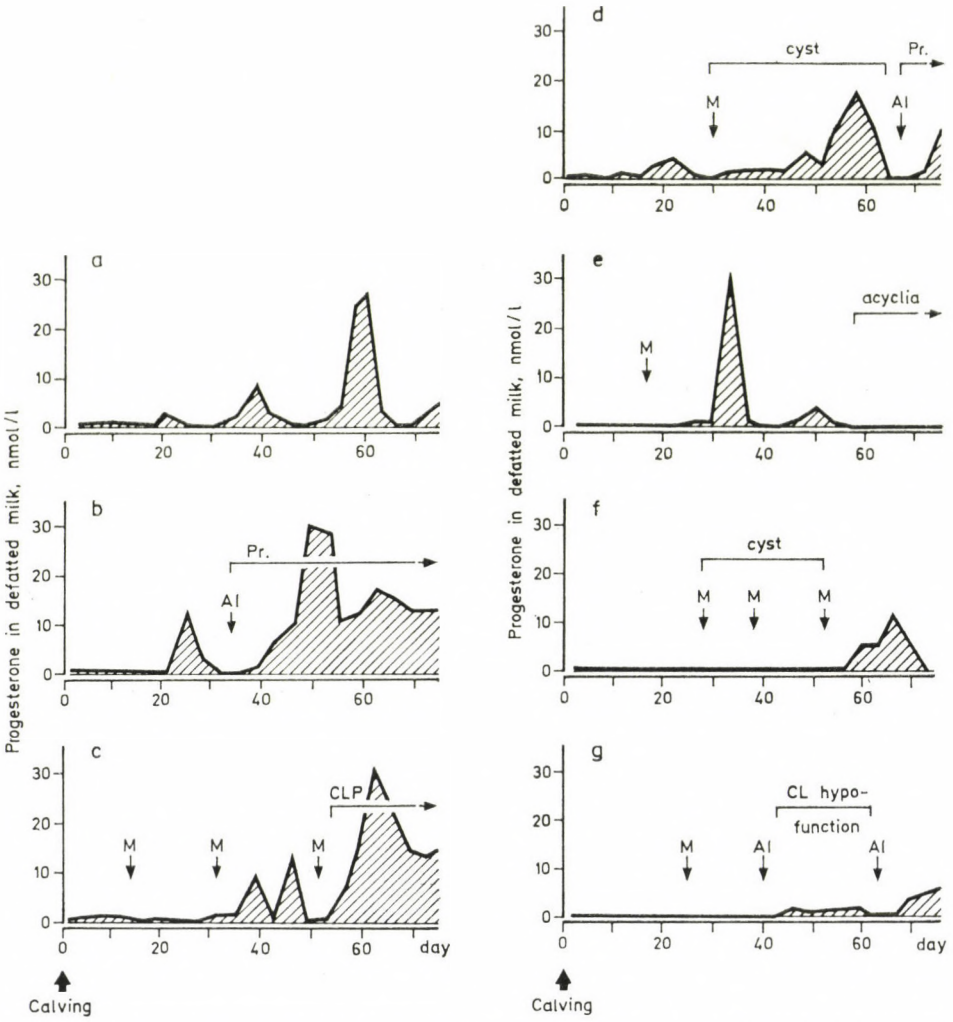

Fig. 2. Typical postpartum progesterone profile curves of healthy animals and of those with pathological ovarian function; *a)* Physiological ovarian reactivation; the cow did not conceive during the examination; *b)* Physiological ovarian reactivation; the cow conceived on day 34; *c)* Corpus luteum persistens; *d)* follicle-lutein cyst; after its spontaneous regression the animal came into oestrus and conceived; *e)* cessation of the once resumed ovarian activity; *f)* delayed resumption of cyclic ovarian activity; a follicle-theca cyst is present during acyclia; *g)* delayed resumption of cyclic ovarian activity followed by CL hypofunction (progesterone max.: 1.6–4.0 nmol/l). Abbreviations: AI = artificial insemination; Pr = pregnancy; M = metritis; CLP = corpus luteum persistens

frequency of cows with large ovarian cysts (Table VII, Fig. 2), was nearly identical in the two groups; cows receiving carotene supplementation developed large ovarian cysts less frequently. The mean of integrates of postpartum progesterone profiles during the first 30 days post partum was practically identical in the two groups; thereafter the treated cows had higher mean values. The dif-

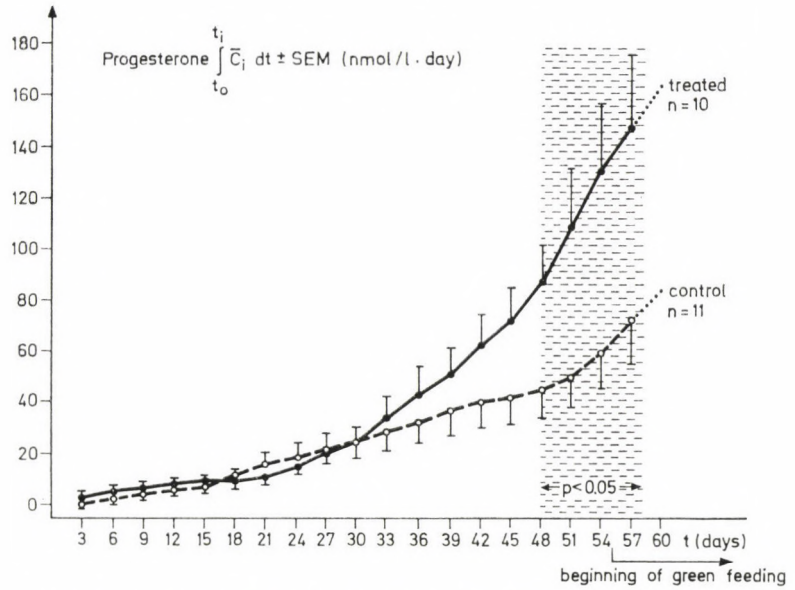

Fig. 3. Group averages of integrates of progesterone profile curves

ference between the two groups in this respect was significant ($P < 0.05$; Fig. 3) from day 48 up to the beginning of green feeding. After the start of green feeding, due to the higher CL-phase progesterone values found for some control cows, the differences were less marked from day 59 onwards; only a tendency remained demonstrable up to the end of the study.

Table VIII

Correlations of certain metabolic parameters with the integrate of progesterone concentration measured up to postpartum day 58

Characteristics of the regression line[1]		Treated[2] (n = 10)	Control (n = 11)	Total (n = 23)
Days 1–5 post partum				
NEFA	A		0.701	
	B		−0.001	
	r		−0.532*	
Total cholesterol	No correlation can be established			
Beta-carotene	A	−5.908		1.677
	B	0.718		0.527
	r	0.608*		0.351*
Days 30–35 post partum				
NEFA	A		0.426	0.398
	B		−0.001	−0.001
	r		−0.561*	−0.317*
Total cholesterol Beta-carotene	No correlation can be established			

Remarks: see Table VI.

Correlations of the integrates calculated for day 58 (the last day for which there were significant differences between the mean integrate of the two groups) with metabolic parameters are shown in Table VIII.

All animals came into oestrus and were inseminated before postpartum day 150. Of them, 7 conceived in each of the two groups.

Although in the treated group the beta-carotene level of the plasma was less than physiological in spite of carotene supplementation, the ratio of cows conceived upon first insemination was more favourable (treated group: 6/7, control group: 1/7) and, consequently, also the conception index was better (treated group: 1.14 $\pm$ 0.35, control group: 2.00 $\pm$ 0.53). The first postpartum inseminations were performed after a mean interval approximately the same in the two groups (control: 65.5 $\pm$ 35.7 days, treated: 64.4 $\pm$ 29.3 days); nevertheless, the treated cows conceived by nearly a cycle-length earlier than the control ones (control: 91.3 $\pm$ 35.6 days, treated: 72.1 $\pm$ 32.4 days).

Discussion

Although the 400-mg carotene supplementation resulted in a significant increase of serum beta-carotene concentration in the treated animals (Fig. 1), the carotene level did not reach 200 μg/dl, the concentration regarded as minimally desirable. A probable explanation for this was the severely carotene-deficient feeding which had been lasting for at least 3 months. Feeding a daily dose of 400 mg beta-carotene, either as a synthetic preparation or a natural feed, is not sufficient to compensate for such a prolonged, severe deficiency. In our earlier experiments with postparturient, high-producing cows similar serum beta-carotene concentrations (between 200 and 400 μg/dl) were achieved by feeding similar daily doses of beta-carotene (Pethes et al., 1983; Huszenicza et al., 1984; Pethes et al., 1984).

Animals characterized by high postpartum NEFA levels were excluded from the evaluation because of the correlation observed between increased lipid mobilization and the delayed resumption of ovarian activity (Haraszti et al., in press), and because of the confirmed relationship between serum NEFA and carotene levels (Haraszti et al., 1984).

As a sign indicative of the resumption of cycling, in the serum and milk of healthy milked cows increased progesterone concentrations were measured for a few days already from postpartum days 16–21. However, there is no consent in the literature as regards the minimum delay of the first postpartum CL phase which can be considered pathological. According to Karg and Schallenberger (1983), a delay of the first postpartum CL phase beyond day 28, whereas according to others beyond day 30 (Van de Wiel et al., 1979), day 35 (Elsaesser et al., 1979; Lamming, 1980), day 45 (Ball et al., 1980) or perhaps day 50 (Lam-

ming and Bulman, 1976; Bulman and Lamming, 1978; Lamming, 1980) post partum can be considered to be a prolonged postparturient anoestrus. The above-cited authors reported wide variations (between 5 and 66%) in the relative incidence of this condition. In the present case, in the treated group the first postpartum CL phase began, on the average, by about 10 days earlier than in the control group; however, due to the large individual variation the difference was statistically non-significant. No correlation was found to exist between the beginning of the first CL cycle and the serum beta-carotene level. At the same time, a low, but significant, negative correlation was found with the NEFA level at postpartum days 1–5 and 30–35 (Table VI). All these facts seem to support that the postpartum resumption of cyclic ovarian activity is delayed by the relative energy-deficiency characteristic of high-producing cows (Eley et al., 1981) and the related enhancement of lipid mobilization (Haraszti et al., 1982; Haraszti et al., in press).

According to the progesterone profile curves, the first CL phase was by some days shorter than the later ones, and was characterized by lower progesterone peaks (Tables IV and VII). This is in agreement with literary data (Morrow et al., 1969; Schams et al., 1978; Peters and Riley, 1982). The duration of the first CL phase was not influenced by the beta-carotene supplementation.

The treated cows tended to have higher progesterone peak concentrations during all the three postpartum CL phases examined than the control cows (Table IV); the differences were not significant. This phenomenon, which, in conformity with the experiences of others (Lotthammer et al., 1976; Jackson et al., 1981) had already been observed by us, may be explained by a postulated direct role of beta-carotene in progesterone synthesis (Pethes et al., 1984). This hypothesis is justified by the fact that carotene-supply-dependent differences were found in the in vitro progesterone secretion, and stimulation thereof with HCG, of surviving CL cell suspensions obtained between days 10 and 12 of the cycle from cows kept on a carotene-deficient diet and from those receiving beta-carotene supplementation (Somorjai et al., 1984; Pethes et al., in press). All these facts explain the significant differences between the two groups in the integrate means of progesterone profile curves from postpartum day 48 up to the beginning of green feeding (Fig. 3). It should be mentioned, however, that the integrate values showed a correlation verifiable by linear regression less frequently with the beta-carotene level than with the NEFA concentration (Table VIII). These call the attention, besides the importance of carotene supply, to the close relationship existing between an adequate energy supply and luteal functions.

The effect of carotene supplementation on the incidence of ovarian disorders characterized by the development of large cysts can be assessed more definitely. Follicle-theca and follicle-lutein cysts were observed in 6 cases altogether (Table VII, Fig. 2). The differences found between the two groups agree

with the experiences of Lotthammer et al. (1976) and Lotthammer and Ahlswede (1977), who reported an increased disposition for the development of large cysts in cows fed carotene-deficient diets. At the same time, our data are consistent with the observations of Zöldág (1984) suggesting that cysts diagnosed shortly after parturition have a high tendency for self-recovery.

The low number and incidence (Table VII, Fig. 2) of the other disorders observed (corpus luteum persistens, cessation of ovarian cyclicity) do not render possible an exact assessment of these conditions. However, carotene supply is unlikely to have an influence on their frequencies.

Because of the low number of animals used and the insufficient basic carotene supply status, the present investigations cannot be considered conclusive. However, based upon the agreement of the present experiences with the conclusions of our previous studies it seems to be plausible that the additional beta-carotene supplementation used in carotene-deficient cows has a favourable effect on postpartum ovarian functions, primarily by decreasing the number of cows with cystic ovaries and by enhancing the progesterone secretion of the CL. However, as compared to the metabolic disturbances resulting from insufficient energy supply, probably the above effect can hardly, or not at all, manifest itself.

Acknowledgements

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STUDIES ON THE LIPID METABOLISM OF EWES IN THE PERIPARTURIENT PERIOD

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The lipid metabolism of eight healthy ewes kept and fed under the conditions of large-scale management technology was studied in the periparturient period.

Blood parameters characterizing lipid metabolism were monitored from the last third of pregnancy up to the third postpartal week. The lipid composition of the triglyceride (TG) and free fatty acid (FFA) fractions of the blood plasma was examined by gas chromatography. At the beginning of the experiment and one week after parturition the total lipid (TL) content and fatty acid composition of liver biopsy specimens were determined. Samples of adipose tissue were taken on three occasions and analysed for fatty acid composition.

Since a half of the sheep proved to be twin-pregnant, it was possible to compare the data of ewes delivering one lamb with those of twinning ewes.

The decrease of the blood glucose level and the increase of its FFA concentration indicated that by the end of pregnancy the ewes, and particularly the twin-pregnant ones, had got into an energy-deficient status. As opposed to dairy cows, in sheep the lower plasma triglyceride level was measured during pregnancy. In the fatty acid composition of triglycerides the proportion of palmitic acid increased while that of stearic acid decreased.

The FFA components were characterized by a change of stearic acid and oleic acid in the opposite direction.

The sheep developed a mild fatty liver during pregnancy. Within the lipids the proportion of stearic acid decreased whereas that of oleic acid increased after lambing.

No important change occurred in the fatty acid composition of the adipose tissue.

Keywords. Lipid metabolism, pregnant ewe, periparturient period.

During pregnancy and in the period around lambing the dam's organism is exposed to an increased metabolic load. Similarly to that of the dairy cow (Haraszti et al., 1982*a*, *b*), the energy and lipid metabolism of the ewe undergoes major changes in this period, with the difference that in sheep these changes occur during late pregnancy, not in the postpartal period (Herriman et al., 1976).

Two factors can be held responsible for the energy-deficiency developing in ewes. Due to the more and more frequent incidence of twin-pregnancy the energy requirement of the fetuses increases. According to Robinson et al. (1978), energy-deficiency can be further aggravated by inappetence which usually accompanies pregnancy.

Of the metabolic changes occurring during pregnancy, besides the decreased lipid synthesis in adipose tissues, Vernon et al. (1981) observed enhanced lipid mobilization. This process is indicated by changes in the concentra-

tion of certain blood plasma metabolites. When the blood glucose level drops, the total lipid (TL) concentration shows a rise. According to Christie (1979) and Noble et al. (1971) the decisive proportion of this rise can be attributed to the increase of the FFA and triglyceride (TG) concentration. Reid and Hinks (1962) found that in late pregnancy the FFA concentration of the blood plasma was directly proportional to the total mass of the fetus(es). According to the studies of Gill and Hart (1980) the peak of lipid mobilization may be estimated to occur on gestation day 135; but this enhanced lipid mobilization continues up to the end of the third postpartal week, i.e. during the period of high milk production. During this period large amounts of ketone compounds are produced in the organism; according to Leng (1965) primarily the level of β-hydroxybutyric acid rises.

Noble et al. (1971) observed in that period characteristic differences also in the fatty acid composition of the blood plasma: the decrease of the proportion of saturated fatty acids, especially stearic acid, and the increase of that of unsaturated fatty acids, were typical.

In ewes, the TL content of the liver, the organ having a paramount importance in energy and lipid metabolism, also increases during pregnancy as it was demonstrated by Gaál and Fekete (1982). This is connected with the increased FFA concentration of the blood plasma (Patterson, 1964). Accordingly, Smith and Walsh (1975) found that the proportion of oleic acid increased and that of stearic acid decreased.

Similarly to fat dairy cows, in obese ewes the lipid mobilization processes are much more expressed and can lead to more severe metabolic changes (Stern et al., 1978) than in normal ewes.

In the present work the data collected on changes in the lipid metabolism of ewes in the period of late pregnancy and early lactation are reported. We investigated the regularities and interactions that exist between some lipid-metabolic parameters of the blood plasma and the fatty-acid composition of adipose tissue and liver parenchyma (the organs having a major role in lipid metabolism) in the above-mentioned period.

Materials and methods

Eight artificially inseminated, 4–5 years old, Merino ewes with an average body mass of 60 kg were used. Four of the ewes delivered twin lambs.

The sheep were fed according to the usual technology of the farm. The daily ration of a ewe consisted of 1 kg alfalfa hay of medium quality, 0.5 kg ewe diet and 0.5 kg barley straw. The ewe diet contained 68% corn-grits, 20% semolina, 10% alfalfa meal, 0.5% mineral supplement, 1% premix supplement and 0.5% fodder salt.

According to calculations, this ration was sufficient to ensure optimum

dry matter, energy and digestible crude protein content in the late pregnancy period and for suckling one lamb.

Follow-up of blood parameters characterizing the lipid and energy metabolism was started 6 weeks before the term (in the last third of pregnancy). Samples were taken weekly up to the third postpartal week. The glucose level of the blood and the total lipid (TL), triglyceride (TG), free fatty acid (FFA) and total cholesterol (TCh) levels as well as aspartate-aminotransferase (AST) activity of the blood plasma were determined. Ketones were demonstrated by the Ross test. The methods applied in the present experiment were the same as those used and described earlier (Husvéth et al., 1982).

To determine their fatty-acid composition, plasma lipid components were separated by thin-layer chromatography. After the extraction of lipids a 5% solution was prepared from the samples with benzene. Two-ml samples of this solution were applied in a band on the preparative thin-layer chromatographic plate made by us (1 mm thick Kieselgel-G on a 20×20 cm glass plate). A 50 : 30 : 20 : 1 mixture of n-heptane, 40% petroleum ether, diethyl ether and acetic acid was used as solvent. The spraying reagent was a 0.5% solution of 2', 7'-dichlorofluorescein in ethanol. The fractions were detected under an ultraviolet lamp of 254 nm wavelength. After scratching off, the fractions were extracted with benzene, evaporated and, after re-measurement, 5% benzene solutions were made from them. Transesterification was carried out as described previously (Husvéth et al., 1982).

The changes in the proportion of fatty acids of TG and FFA fractions which presumably play a decisive role in the alteration of lipid composition were studied by a gas-chromatographic procedure (Husvéth et al., 1982).

On two occasions, at basic sampling at the beginning of the experiment (A) and one week after lambing (+1) about 100 mg liver tissue was obtained by percutaneous needle biopsy. The TL content of the samples was determined by a chemical method (Atkinson et al., 1972). In the extracted lipids the proportion of individual fatty acids was studied by gas chromatography.

Adipose tissue samples were taken from the subcutaneous connective tissue of the tailhead. At the beginning of the experiment (A), one week before (−1) and after lambing (+1) about 500 mg fat was excised on each occasion (Johnson et al., 1977). The fatty-acid composition of adipose tissue lipids was analysed similarly to that of the liver samples.

Our results were analysed by statistical methods (Antal et al., 1978). Data obtained 6 and one week before and one week after parturition were subjected to significance and linear regression calculations. This approach was chosen because, as compared to the basic samples, the most striking changes occurred at the above times.

Blood parameters examined at the above three times (A, −1, +1) in ewes delivering one lamb were compared with those of the twin-pregnant ewes.

Results

Analysis of blood samples

Results of the analysis of blood samples are shown in Table I.

One week before parturition the blood glucose level dropped significantly, by about 20%. In the first week of lactation it approximated the lower limit of the physiological level found at the beginning of the experiment and measured by us in non-pregnant ewes (Fig. 1).

The TL, TG and FFA levels of the blood plasma rose during the study.

By week −1 the TL had reached a level one and a half times as high as the base value, and remained at the same level after lambing.

TG concentration was well below the physiological range during pregnancy; however, by one week before parturition it had increased by 30% and continued to rise even after lambing. In the third week of lactation it exceeded the base level already by 80%.

At the beginning of the experiment the FFA level was 0.42 mmol/l, which corresponds to the upper limit of the physiological range. By one week before lambing it had increased by a further 30% and dropped to the mean level in the third week of lactation.

TCh was low throughout the period of study (it was mostly around 2.6 mmol/l). At the first sampling and immediately after lambing it decreased by a further 20%.

AST activity remained within, or slightly above, the physiological range throughout the period of study.

Of the blood parameters, significant differences were demonstrated in glucose, FFA and TCh levels between uniparous and twinning ewes (Fig. 2). The glucose concentration of the basic sample was 30% lower, and one week before lambing FFA level was 60% higher in the twinning ewes.

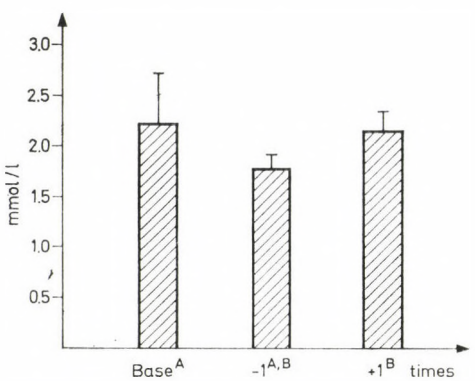


Fig. 1. Changes in blood glucose level at the three times studied (values indicated with the same letter differ significantly [A, B: $P < 0.001$]; A = basic sampling; −1: one week before parturition; +1: one week after parturition)

Table I

Changes in total lipid, triglyceride, free fatty acid, total cholesterol levels and AST activity of the blood plasma

		Base	−5	−4	−3	−2	−1		+1	+2	+3
		Weeks before parturition							Weeks after parturition		
TL, g/l	$\bar{x}$	1.98[A, B]	2.03	2.06	2.03	2.60	*2.96[A]	PARTUR-	2.94[B]	2.19	2.38
	±s	±0.31	±0.25	±0.28	±0.27	±0.55	±0.85	ITION	±0.62	±0.31	±0.35
TG, mmol/l	$\bar{x}$	+, ++0.144[C, D]	0.140	0.139	0.134	0.134	0.178[C]		0.195[D]	0.230	0.256
	±s	±0.039	±0.060	±0.048	±0.034	±0.023	±0.021		±0.043	±0.044	±0.035
FFA, mmol/l	$\bar{x}$	0.42[E]	0.48	0.37	0.37	0.44	*0.56[E]		0.45	0.44	0.35
	±s	±0.05	±0.16	±0.09	±0.07	±0.09	±0.17		±0.06	±0.09	±0.14
TCh, mmol/l	$\bar{x}$	+2.16[C]	2.84	2.71	2.60	2.80	2.67[C, D]		2.12[D]	2.19	2.64
	±s	±0.48	±0.80	±0.86	±0.97	±1.06	±0.39		±0.50	±0.49	±0.40
AST, U/l	$\bar{x}$	++30.3	51.7	52.6	51.2	42.0	37.0		47.3	52.9	67.7
	±s	±4.3	±12.6	±19.2	±13.6	±10.6	±10.0		±17.5	±15.9	±23.1

* Positive correlation: $r = 0.74$; $P < 0.05$; +, ++ Negative correlation: + $r = -0.71$; $P < 0.005$; ++ $r = -0.78$; $P < 0.05$. Parameters indicated with the same letter in the horizontal lines differ significantly: A, B: $P < 0.001$; C, D: $P < 0.05$; E: $P < 0.01$.

Table II

Changes in the proportion of free fatty acid (FFA) components in the blood plasma

		FFA, mmol/l	Pentadecanoic acid C15 : 0	Palmitic acid C16 : 0	Palmitoleic acid C16 : 1	Stearic acid C18 : 0	Oleic acid C18 : 1	Linoleic acid C18 : 2	Arachidonic acid C20 : 0	Linolenic acid C18 : 3
A	$\bar{x}$	0.42+, 1	0.54	22.38[1]	2.66	35.42	27.56	7.43	0.74	3.33
	±s	±0.05	±0.27	±2.20	±0.55	±3.71	±3.72	±1.17	±0.57	±0.98
−1	$\bar{x}$	0.56[2,+]	0.91	22.08	2.73	33.21	28.67[2]	6.45	0.48	2.57
	±s	±0.17	±0.50	±2.67	±1.07	±2.56	±5.82	±3.21	±0.21	±0.59
+1	$\bar{x}$	0.45	0.66	21.40	3.86	33.49	31.40	7.39	0.55	2.02
	±s	±0.06	±0.49	±2.94	±3.71	±2.83	±4.90	±1.00	±0.52	±0.51

A: Basic sampling; −1: one week parturition; +1: one week after parturition; 1: positive correlation ($r = 0.90$; $P < 0.01$); 2: positive correlation ($r = 0.88$; $P < 0.01$); +: significant difference $P < 0.01$.

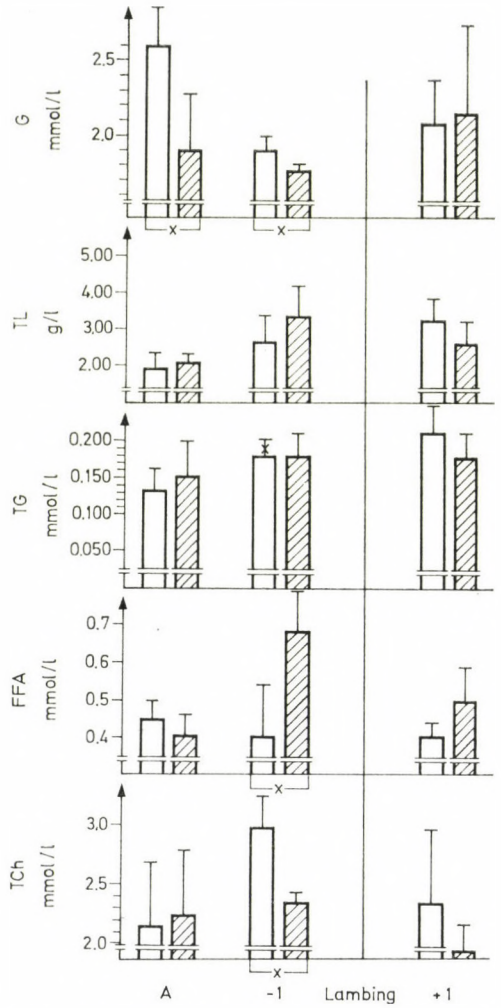


Fig. 2. Differences in blood parameters between uniparous and twinning ewes (white columns: uniparous ewes, striated columns: twinning ewes; ⌊x⌋: $P < 0.05$)

In the basic samples, the TG and TCh level and the TG level and AST activity were in negative correlation with one another. One week before lambing a positive correlation was found between TL and FFA (Table I).

Of the FFA components, the proportion of stearic acid (C18:0) decreased, whereas that of oleic acid (C18:1) increased during the experiment. In the basic samples a positive correlation was established between the FFA level and the proportion of palmitic acid (C16:0). At one week before parturition, on the other hand, the FFA level was in a close correlation with oleic acid (Table II).

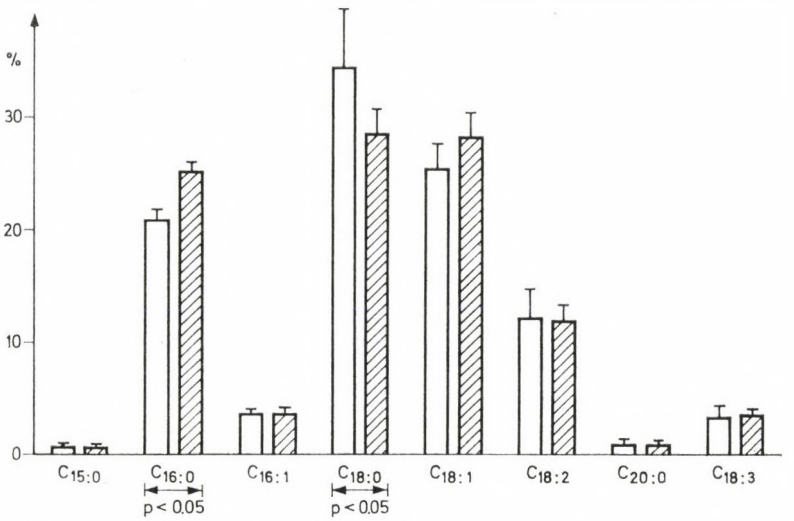
In the TG fraction of the blood plasma the proportion of palmitic (C16:0) and palmitoleic acid (C16:1) increased, and that of stearic acid (C18:0) de-

creased one week after lambing. The changes were statistically significant. As compared to the base value, the proportion of linoleic acid (C18:2) was significantly higher already before parturition (Table III).

Analysis of liver samples

The results obtained by the analysis of liver samples are shown in Fig. 3.

At the beginning of the last third of pregnancy, the TL concentration of the liver was, on the average, 77 g/kg. As compared to this, TL showed an about 30%, statistically significant ($P < 0.05$), decrease after lambing.



$\bar{x}$	0.53	0.43	20.67	23.15	3.49	3.48	34.24	28.78	25.39	28.45	11.97	11.84	0.78	0.78	3.01	3.09
±s	0.17	0.08	1.44	1.32	0.68	0.33	5.38	2.75	2.57	3.50	3.39	1.42	0.69	0.26	1.32	0.75

Fig. 3. Total lipid concentration of liver biopsy specimens and proportions of the different fatty acids (white columns: Base^A = 77 ± 14.4 g/kg; striated columns: +1^A = 53.6 ± 5.8 g/kg; A: $P < 0.05$)

As regards the fatty acid composition of hepatic lipids, in the basic samples stearic acid (34.34%), oleic acid (25.39%), palmitic acid (20.67%) and linoleic acid (11.97%) were present in the highest proportions.

At week +1 (one week after parturition) stearic acid and oleic acid were present in the liver samples in nearly identical proportions (28.74 and 28.45%, respectively), indicating that the proportion of stearic acid had decreased while that of oleic acid had increased. The proportion of palmitic acid also showed a slight rise (23.15%).

Hepatic TL showed no correlation with any of the fatty acids tested.

Table III

Changes in the proportion of fatty acid components of triglycerides in the blood plasma

		TG, mmol/l	C15 : 0	C16 : 0	C16 : 1	C18 : 0	C18 : 1	C18 : 2	C20 : 0	C18 : 3
A	$\bar{x}$	$0.144^{A, B}$	1.81	24.92^{C}	5.21^{A}	35.21^{A}	22.27	$4.18^{A, B}$	1.08	4.55^{C}
	$\pm s$	± 0.039	± 0.62	± 2.87	± 1.41	± 6.81	± 2.06	± 1.62	± 0.70	± 1.45
−1	$\bar{x}$	0.178^{A}	2.61	26.87	7.09	32.03	22.65	6.11^{A}	0.76	1.79^{C}
	$\pm s$	± 0.021	± 1.57	± 2.80	± 3.77	± 9.30	± 3.32	± 1.06	± 0.19	± 0.22
+1	$\bar{x}$	0.195^{B}	2.49	28.70^{C}	7.54^{A}	27.16^{A}	23.92	5.95^{B}	0.63	3.20
	$\pm s$	± 0.043	± 1.09	± 4.22	± 2.69	± 3.91	± 4.82	± 1.52	± 0.16	± 1.57

A: Basic sampling; −1: one week before parturition; +1: one week after parturition. The values indicated with the same letter within a column differ significantly from one another: A, B: $P < 0.005$; C: $P < 0.01$.

Table IV

Fatty acid composition of adipose tissue in the period studied

		C14 : 0	C15 : 0	C16 : 0	C16 : 1	C18 : 0	C18 : 1	C18 : 2	C20 : 0	C18 : 3
A	$\bar{x}$	3.18	0.54	26.34	2.62	22.96	39.42	3.06	0.47	1.20
	$\pm s$	± 0.63	± 0.13	± 1.77	± 0.83	± 4.99	± 4.81	± 0.55	± 0.28	± 0.30
−1	$\bar{x}$	3.20	0.68	26.22	2.47	24.13	38.94	2.84	0.47	1.04
	$\pm s$	± 0.49	± 0.24	± 2.33	± 0.56	± 6.99	± 5.89	± 0.25	± 0.10	± 0.20
+1	$\bar{x}$	2.93	0.43	25.85	2.63	23.05	40.12	3.14	0.62	1.06
	$\pm s$	± 0.25	± 0.20	± 2.33	± 0.58	± 4.50	± 4.10	± 0.54	± 0.20	± 0.27
		N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

A: Basic sampling; −1: One week before parturition; +1: one week after parturition.

Analysis of subcutaneous fat

For results of the analysis of subcutaneous fat, see Table IV.

Nine fatty acids were separated from the adipose tissue. In the basic samples three fatty acids, viz. oleic acid, palmitic acid and stearic acid (in the order of their proportion) were present in the highest proportion. The proportion of the remaining fatty acids was negligible, at most a few per cent. Fatty acid composition remained practically unaltered throughout and no significant difference from the basic sample was observed.

Discussion

The lipid metabolism of ewes kept under large-scale management and fed as usual was investigated in the period of pregnancy. Certain blood parameters characteristic of lipid metabolism and the composition of hepatic and adipose tissue were monitored from the beginning of the last third of pregnancy up to the peak of lactation. Four of the eight ewes delivered twin lambs, thus, it was possible to compare the results obtained in uniparous and twinning ewes.

The results allowed us to conclude that, despite the fact that the calculations indicated a sufficient energy supply, by the end, and particularly in the last week, of pregnancy the ewes had developed an energy-deficient status as indicated by the decreased blood glucose and increased FFA levels. Unlike Gill and Hart (1980), we could not demonstrate a rise of the FFA level in the third week of lactation. Neither the ewes pregnant with one fetus nor the twin-pregnant ones showed a pathological increase of ketone concentration; however, the significantly lower blood glucose and higher FFA level of twin-pregnant ewes indicated a higher energy requirement of twin fetuses from the dam's organism, in accordance with the observations of Robinson et al. (1978). Only at week −1 and week +1 did the TL content of the blood plasma exceed, by about 50%, the 1.63–2.24 g/l level reported by Garton and Duncan (1964) to be physiological in sheep. At the beginning of the experiment, the TG concentration was subnormal, then it rose continuously. This finding is inconsistent with the observations made in cattle; namely, in non-lactating cows the TG level of the blood plasma is always higher than in lactating ones (Henricson et al., 1977).

The negative correlation established between TCh and TG may be due to the fact that in the advanced stage of pregnancy the capacity of the liver is devoted, within lipid metabolism, primarily to TG formation (Puppione, 1978). In cattle, the drop of TCh concentration can be attributed to its disturbed hepatic synthesis (Brumby et al., 1975), which holds true also for sheep. It is characteristic that before parturition the greatest influence on blood plasma TL is exerted by the rise of FFA concentration, since in this period, due to the increased lipid mobilization, FFA attains to an important role (Christie, 1979).

In the period studied, alterations in the fatty acid composition of blood plasma lipids can be characterized better by the proportions of fatty acids constituting TG and FFA; therefore, instead of the analysis of TL, only TG and FFA were subjected to gas-chromatographic analysis.

The proportion of palmitic acid within FFA was consistent with the results of West and Annison (1964). Adipose tissue contains mostly oleic acid; thus, as a consequence of the increased preparturient lipolysis it was natural that within plasma FFA primarily the proportion of oleic acid increased (Noble et al., 1971). The concentration changes of stearic acid and oleic acid were opposite in ewes, similarly to what was observed for cows (Husvéth et al., 1982).

The composition of TG was characterized by an increase in the proportion of palmitic acid and a decrease in that of stearic acid.

In sheep, endogenous fat depots exhibit a high activity; however, during rapid and considerable lipid mobilization the subcutaneous fat has the most important part (Ingle et al., 1972). Therefore, the subcutaneous fat of the tail-head area, from where samples of fat can be taken easily and in large masses, was considered to be most suitable for fat sampling. No appreciable change was found in the composition of adipose tissue, indicating that after lipolysis fatty acids of different C atom numbers entered the blood circulation. Because of the absence of glycerokinase, no re-synthesis of lipids occurs in the adipose tissue (Khachadurian et al., 1967).

Hepatic TL did not exceed 100 g/kg, the level considered to be the upper limit of the physiological range in the peripartal period in cows. However, the mean value measured before parturition was more than 1.5 times the value found for non-pregnant animals (Magdus et al., unpublished observation). In the liver of sheep suffering from pregnancy toxaemia, by a histological method Patterson (1966) measured a lipid content of 9 to 25%. Comparing the individual values to those of the table elaborated for cattle to allow comparison of histological and biochemical data (Gaál et al., 1983), it can be established that the histological result indicates a mild fatty liver. In the present experiment, although pregnancy toxaemia did not develop, a mild lipid accumulation was demonstrated in the liver on the basis of the chemical examination. This phenomenon is similar to the postparturient fatty liver developing in high-producing dairy cows, with the difference that in ewes the fatty liver developed before lambing.

In the hepatic tissue, of the fatty acids with a long carbon chain the decrease of stearic acid and the increase of oleic acid were consistent with the findings of Smith and Walsh (1975). These changes are identical with those observed in dairy cows after parturition (Husvéth et al., 1982). Similar differences were measured in the degree of changes of plasma FFA, stearic acid and oleic acid values. In sheep, however, the proportion of stearic acid decreased mainly in TG and that of oleic acid remained practically unaltered.

Unlike other investigators (Patterson, 1964) we could not confirm any correlation between hepatic TL and blood plasma FFA concentration. This can be explained by the fact that in the present case no severe lipid accumulation occurred in the liver.

According to the parameters studied, the lipid metabolism of ewes undergoes changes, similarly to those found for dairy cows (Haraszti et al., 1982; Husvéth et al., 1982). This occurs also in the case of an energy supply considered sufficient, and reaches a particularly severe degree in twin-pregnant ewes. In ewes, however, metabolic changes reach the peak in the week immediately preceding parturition.

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EFFECT OF LONG-TERM DEHYDRATION ON THE BODY WEIGHT, BODY TEMPERATURE AND PACKED CELL VOLUME IN BUFFALO*

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In order to study the effect of dehydration on body weight (b. wt.), body temperature (b. temp.) and packed cell volume (PCV) in buffalo, 6 buffalo calves kept under normal conditions and 9 kept on long-term dehydration, all aged 1 to 1.5 years, were used. The animals at long-term dehydration clinically showed cachectic and soporous behaviour, shrunken eyes, anorexia, eye discharge, hard faeces and uncoordinated gait. The buffalo calves survived maximum 15 days of dehydration. The values of b. temp. and PCV in normal buffalo calves ranged between 37.9 and 39.0 °C and between 28 and 43%, respectively. There was significant ($P < 0.01$) reduction in b. wt., increase in PCV and fall in b. temp. from day 3, 6, and 12 onwards, respectively.

There was significant ($P < 0.05$) negative correlation between PCV and relative humidity (rel. hum.) and between PCV and b. temp., and positive correlation between b. wt. and b. temp. Multiple correlation studies showed significant ($P < 0.01$) correlation of b. wt. with PCV, rel. hum. and b. temp., and of PCV with b. wt., rel. hum. and b. temp. It is apparent that also the rel. hum. in the sheds played an important role in the regulation of water metabolism.

Keywords. Dehydration, body weight, body temperature, packed cell volume, buffalo.

The clinical symptoms appearing due to dehydration have been reported in the camel (Ghoshal et al., 1973), cow and horse (Duncan and Prasse, 1977) and in domestic animals in general (Blood et al., 1979). The data during long-term dehydration are available on the various physiological changes in cattle (Weeth et al., 1967, 1970; Schalm et al., 1976; Blood et al., 1979), sheep (Schmidt-Nielsen, 1956) and camel (Schmidt-Nielsen, 1956; Ghoshal et al., 1973). No such record is available in the buffalo, an animal of great importance as high milk yielder. Therefore, the present work was undertaken.

Materials and methods

Fifteen healthy male buffalo calves aged 1 to 1.5 years were used. Six control animals were kept under ideal farm conditions within closed shed, fed on green berseem, maize choff fodder and water *ad libitum* for at least 7 days.

**Editor's note:* The present article is published with all due reserves as regards the methods used by the authors. The Editorial Board deem it necessary to express their disapproval of the applied methods, with regard to reasons of animal protection. However, for the lack of relevant data on long-term dehydration in buffalo, the publication of this paper has been considered justifiable.

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Nine experimental animals were initially kept for at least 2 days under similar conditions to acclimatize. Subsequently, water was completely withdrawn and only dry wheat straw was given to induce long-term dehydration. As recorded on two buffalo calves, they could not survive the 15th day of dehydration. This served as landmark for lethal stage of dehydration and hence, on other animals the observations were recorded for 12 to 14 days of dehydration.

The body weight (b. wt.), body temperature (b. temp.) and packed cell volume (PCV) were recorded in normal animals and on days 1, 3, 6, 9 and 12 of dehydration in experimental animals. Clinical symptoms in these animals and the relative humidity (rel. hum.) of the animal shed were recorded during the entire period of study. The data between animals and between days of dehydration were statistically analysed (Snedecor and Cochran, 1967). The correlation between b. wt., b. temp., PCV and rel. hum. and multiple correlation of b. wt. versus b. temp., PCV and rel. hum., and of PCV versus b. wt., b. temp. and rel. hum. were also calculated.

Results

The clinical symptoms of the normal and the experimental buffalo calves on the 1st day of experiment included bright eyes, soft skin of normal turgor, moist muzzle, normal gait and soft faeces. Clinical symptoms recorded on experimental animals during total deprivation of water were: (a) gradual anorexia from day 4; (b) shrunken eyes from day 3; (c) cachectic and soporous behaviour

Table I

Mean and range (in parentheses) values of body weights, packed cell volume and body temperature in buffalo calves and of relative humidity on different days of long-term dehydration, in comparison with control animals

Days of observation	Relative humidity (%)	Body weight (kg)	Packed cell volume (%)	Body temperature (°C)
Control animals	71.00 (59.00–85.00)	94.00 (66.00–154.00)	32.33 (38.00–43.00)	38.36 (37.88–39.00)
1	67.55 (51.00–85.00)	93.77 (81.00–110.00)	35.33 (28.00–47.00)	38.95 (37.88–39.22)
3	70.05 (54.50–85.00)	79.11 (64.00–98.00)	37.44 (30.00–47.00)	38.29 (37.77–39.44)
6	70.58 (59.33–85.00)	72.00 (58.00–82.00)	38.44 (32.00–48.00)	38.33 (37.66–39.22)
9	67.85 (57.00–85.00)	66.11 (54.00–76.00)	39.56 (33.00–48.00)	38.03 (37.22–38.77)
12	67.96 (55.33–85.00)	61.33 (49.00–71.00)	40.33 (32.00–59.00)	37.75 (36.80–38.44)
CD between parameters on different days at the $P < 0.05$ level		2.92	2.36	0.43

Table II

Analysis of variance for body weight, packed cell volume and body temperature in buffalo calves

	Source of variation	df	MS
Body weight	Between days	4	1446.4667**
	Between animals	8	441.2000**
	Error	32	9.2417
PCV	Between days	4	34.2778**
	Between animals	8	176.1722**
	Error	32	6.0403
Body temperature	Between days	4	0.7300*
	Between animals	8	0.4727*
	Error	32	0.2005

* Significant at the $P < 0.05$ level.
** Significant at the $P < 0.01$ level.

Table III

Correlation and multiple correlation coefficients between variables in dehydrated buffalo calves

Variables	Correlation coefficient	Variables	Multiple correlation coefficient
b. wt. vs PCV	−0.1782	b. wt. vs PVC, rel. hum. and b. temp.	0.3955**
b. wt. vs b. temp.	0.3820*	PCV *e* b. wt., rel. hum. and b. temp.	0.4481**
PCV vs b. temp.	−0.2052*		
PCV vs rel. hum.	−0.4589*		
b. temp. vs rel. hum.	0.1350		

* Significant at the $P < 0.05$ level.
** Significant at the $P < 0.01$ level.

from day 2 to 8; (d) eye discharge and slight frothing at the mouth from day 4 to 10; (e) hard faeces from day 4; (f) stiff and uncoordinated gait with recumbency from day 10.

The mean and range values of b. wt., b. temp., and PCV in control animals and in experimental animals during the dehydration period and the relative humidity in the shed are presented in Table I. The analysis of variance of the data on b. wt., b. temp. and PCV (Table II) indicated significant differences between days as well as between experimental animals ($P < 0.01$). Significant reduction ($P < 0.01$) in b. wt. was observed from day 3 onwards. Body temperature decreased significantly ($P < 0.05$) from day 12 onwards and PCV increased ($P < 0.01$) from day 6 onwards (Table I).

The data have shown negative nonsignificant correlation between b. wt. and PCV, PCV and b. temp. and a significant negative correlation between PCV and rel. hum. A significant positive correlation existed between b. wt. and b. temp., while a positive nonsignificant correlation appeared between b. wt. and rel. hum. and also between b. temp. and rel. hum. (Table III).

The multiple correlation of b. wt. versus b. temp., PCV and rel. hum. and of PCV versus b. wt., b. temp. and rel. hum. was highly significant ($P < 0.01$; Table III).

Discussion

The clinical symptoms shown by the control and experimental buffalo calves were similar to those described in camel (Ghoshal et al., 1973), cow and horse (Duncan and Prasse, 1977) and in other domestic animals (Blood et al., 1979). The fluid content of the body is dependent upon a delicate balance between intake and output. However, the sensation of thirst seems to be related with the electrolyte concentration of the serum or intracellular fluid. Bianca et al. (1965) and Mac Farlane et al. (1961) found that total deprivation of water caused an initial increase of plasma and extracellular volume with a decrease during later period along with plasma proteins. A significant rise in PCV values during progressive dehydration in cows has been reported by Schalm et al. (1976); however, Henschel (1964) did not observe any change in haemoconcentration during long-term dehydration. The plasma volume is increased through the absorption of water from the gastrointestinal tract leading to hardening of faeces, which is evident in buffalo calves from day 4. Also the diffusion of extracellular fluid into blood volume must have resulted in the dryness of skin and shrinking of the eyeball. The stiff and uncoordinated gait with recumbency from day 10 suggests an increase in the electrolyte concentration leading to acidosis and stiffening of the muscles.

The increased concentration of plasma electrolytes might have stimulated continuously the hypothalamic centres, resulting in anorexia. Consequently, a reduction of metabolism and b. wt. resulted. In buffalo calves significant reduction of b. wt. and increase in PCV appeared from day 3 and 6, respectively. The significant variation in the b. wt. among the various days of starvation, particularly during the later days, is indicative of the fact that body metabolism responsible for heat production has been reduced to minimum due to stoppage of nutrients to experimental animals. At the same time body reserve fat was utilized to generate heat and to maintain the b. temp. of the experimental animals. It favours the decline in b. wt. of these animals. The decline contributed about 33.4 to 39.5% loss of b. wt. in experimental animals. Also, it was observed that a loss of 38% b. wt. was fatal in dehydrated animals. An animal died on the 15th day after attaining a stage of 38% loss in b. wt. when the rel. hum.

was 72.0%, while another animal died with a loss of 35.6% b. wt. when the rel. hum. was 42%. So it could be concluded that days of starvation contributed significantly ($P < 0.01$) to progressive loss of b. wt. between the days (Table II). The loss of b. wt. between the experimental animals was significant ($P < 0.01$) due to individual differences in genetic potential (Table II). Schmidt-Nielsen (1956) indicated 30% loss in b. wt. during summer to be fatal in Merino sheep and camels. Houpt (1970) and Blood et al. (1979) indicated 10 to 12% reduction in b. wt. during severe dehydration in domestic animals. Weeth et al. (1967, 1970), however, reported 16% loss in b. wt. in cattle after 4 days of dehydration. There was a significant ($P < 0.05$) decrease in b. temp. in the experimental animals from day 12 onwards. This decrease appears to be due to most unfavourable conditions in terms of non-availability of nutrients, dysfunction of the neurohumoral mechanism resulting in a considerable decrease or complete cessation of heat generation from cell metabolism during later stages of dehydration. Also the environmental heat, water restriction and rel. hum. might result in the lowered b. temp. as suggested by Rosenmann and Morrison (1967) in reindeer. In camel, Schmidt-Nielsen (1965) and Ghoshal et al. (1973) reported a variation of 6 °C and 1 °C in b. temp. during summer due to dehydration.

The above facts are further supported by the present study indicating a significantly positive correlation between b. wt. and b. temp. and a significant negative correlation between PCV and b. temp., and between PCV and rel. hum. Furthermore, while no significant correlation was found between b. wt. and PCV and between b. temp. and rel. hum., there existed significant multiple correlation coefficients of b. wt. with PCV, rel. hum. and b. temp., and of PCV with b. wt., rel. hum. and b. temp.

Thus, it is evident that during dehydration, b. wt., b. temp. as well as PCV are affected, and PCV values are well correlated with changes in both the b. temp. and rel. hum., while the latter two are not interdependent.

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EFFECT OF MYCOPLASMA CONTAMINATION OF BULL SEMEN ON FERTILIZATION

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Semen samples from bulls were tested for mycoplasma by culturing. Reproductive-biological indices (ratio of not fertilizing to successfully fertilizing semen doses calculated for the conceived heifers; the same ratio for the whole heifer stock; ratio of heifers conceived after more than one insemination to those conceived at the first attempt; ratio of non-conceived heifers to conceived ones; ratio of semen doses failing to fertilize upon the first attempt to those successfully fertilizing at the first attempt, in the conceived group; the same ratio for the whole heifer stock) for two bulls with mycoplasma-negative semen were compared to those of 4 mycoplasma-excreting bulls, in four farms. Statistical evaluation of the results was performed by the chi-squared (χ^2) test. In addition, a comparison was made between heifers inseminated with mycoplasma-infected and uninfected semen by fitting (χ^2 contingency) in respect of their distribution according to the number of insemination attempts. The relative incidence of semen doses resulting in acyclia was also compared. The results are suggestive of the aetiological role of mycoplasma infection and the economic losses due to the use of infected bull semen.

Keywords. Bull semen, *Mycoplasma*, fertilization.

The profitability of cattle breeding largely depends on reproductive performance, which is influenced by several factors, viz. genetic properties, feeding, managemental hygiene, semen quality, contamination of semen with infectious agents, etc. Both the investigations conducted in Hungary (Stipkovits et al., 1983) and foreign data indicate that the bull semen used in the practice frequently contains mycoplasma.

From the bovine genital tract *Mycoplasma* organisms were first isolated in 1947 (Edward et al., 1947). The isolates belonged to two groups: a pathogenic (P) and a saprophytic (S) group. Later on the P strains were identified as *Mycoplasma bovigenitalium*, while the S strains as *Acholeplasma laidlawii*. Subsequently, other *Mycoplasma* species were also found to occur in the urogenital tract of both cows and bulls as well as in bull semen. For lack of extensive experimental studies, the role of most of these species in urogenital disorders has remained unclear. Recent studies have revealed the pathogenicity of certain *Mycoplasma* species. In addition to their role played in the aetiology of bovine urogenital diseases, these species have been incriminated as possible causative agents of reproductive-biological disorders.

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More than 10 *Mycoplasma* species are known to have a role in bovine urogenital mycoplasma infection. The following species occur most frequently: *M. bovigenitalium*, *M. bovis*, *A. laidlawii*, and *Ureaplasma diversum*. With the exception of *A. laidlawii*, all these species have been proved to be pathogenic. Although *A. laidlawii* is frequently isolated (Hoare, 1969; Pan and Ogata, 1969), its pathogenicity is uncertain (Gourlay and Howard, 1979). Mycoplasmas were cultured from the prepuce, urethra and semen of bulls very frequently (Taylor-Robinson et al., 1969; Onoviran et al., 1975; Langford, 1983; Jurmanova and Mazurova, 1978; Jurmanova et al., 1983). Of these *Mycoplasma* species *M. bovigenitalium*, *M. bovis* and *U. diversum* are of outstanding importance. Mycoplasmas can survive in frozen semen for months (Hirth et al., 1967). However, mycoplasma infection of the semen and of the lower genital tract is not always accompanied by clinically apparent disease. Infection of the upper genital tract by *M. bovigenitalium* and *M. bovis* usually resulted in seminal vesiculitis and, less frequently, in epididymitis (Ernø and Blom, 1972). Experimental induction of the disease was successful either by injecting the above *Mycoplasma* species into the seminal vesicle (Parsonson et al., 1974; Panangala et al., 1982) or by experimental infection with ureaplasmas (Waelchli-Suter et al., 1982; La Faunche and McEntee, 1982). Infection of the genital tract usually did not alter spermatogenesis, however, the maturation process of spermatozoa was disturbed by the presence of mycoplasmas in the semen. This was indicated by an increased number of dead or morphologically abnormal spermatozoa in the infected semen samples (Holzmann et al., 1982), a reduced motility of spermatozoa, particularly after deep-freezing (Jurmanova and Sterbova, 1977; Panangala et al., 1982), and by a lower glutamic acid, tyrosine and total protein content of the semen (Ibrahim et al., 1984). Each of these factors may serve as a source of reproductive-biological disturbances. In the present work, we investigated the relationship between the mycoplasma-contamination of the semen used for insemination and the conception of heifers.

Materials and methods

Straws, each containing a semen sample from one bull, were opened in a sterile manner, and their contents were inoculated into 2 ml liquid BEG or Livingston's medium (Ernø and Stipkovits, 1973; Livingston, 1972). The cultures were incubated at 37 °C for 10 days. On the 2nd, 5th and 10th days of incubation subcultures were made on solid media of the same type. The agar plates were incubated at 37 °C in the presence of 5% CO_2. Every two days, the plates were examined for the presence of classical mycoplasmas and ureaplasmas under a colony-counting microscope. As mycoplasma colonies had appeared, agar blocks approximately 5×10 mm in size were transferred into a

liquid medium for further passage. The resulting subcultures were filtered through a 450-nm filter, cloned, and the cultural and biochemical properties (glucose- and urea-splitting and arginine-hydrolysing capacity, phosphatase production, triphenyltetrazolium chloride (TTC) reduction, film and spot production; Stipkovits, 1977) of the strains were studied. In addition, the classical mycoplasmas were examined by the growth-inhibition test. Antisera produced in this laboratory against the 17 bovine *Mycoplasma* and *Acholeplasma* strains (Stipkovits and Varga, 1974) were used.

Semen samples from 18 bulls were examined. The bulls were kept at different artificial insemination (A. I.) stations; all of them were clinically healthy and showed satisfactory semen production.

Six Black and White Holstein-Friesian bulls were used for the evaluation of reproductive-biological results; the semen of 4 of these bulls contained mycoplasmas, whereas from that of the two remaining bulls we failed to culture mycoplasma.

Evaluation of the bulls was performed on the basis of conception data for heifers inseminated in different farms, taking into account the number of inseminations per heifer and whether the semen did or did not contain mycoplasma. The semen doses applied in each heifer originated from the same bull.

The following reproductive-biological indices were used:

(1) the ratio of not fertilizing to successfully fertilizing semen doses (a) calculated for heifers having conceived, and (b) the same ratio calculated for the whole stock;

(2) the ratio of heifers conceived after more than one attempt to those conceived at the first attempt;

(3) the ratio of non-conceived to conceived heifers;

(4) the ratio of semen doses failing to fertilize at first attempt to those successfully fertilizing already at the first attempt (a) for heifers having conceived, and (b) the same ratio for the whole stock;

(5) the ratio of semen doses resulting in acyclia to those resulting in fertilization for the conceived heifers; and

(6) the distribution of heifers conceived after one, two, three, etc. attempts.

Significance calculations were performed for all parameters by the χ^2 test.

The above indices were formed to allow an easy implementation of the χ^2 test; therefore, they slightly differ from the indices usual in cattle breeding (pregnancy index, fertilization index, conception %, etc.).

The role of mycoplasma infection was studied by comparing the reproductive-biological results of infected and uninfected bulls; for this purpose, the inseminations were performed on the same farm, within farm in the same unit and at the same time.

Results

The comparison was made on four different farms.

Progeny-testing results of infected and uninfected bulls

The data were collected from sire catalogues of A. I. stations. Table I shows that the infected bulls were inferior to the uninfected ones as regards the number of progeny-tested offspring and milk and butterfat production; there were no differences in the other parameters.

Table I

Progeny-testing results of the bulls used in the study

Designation of bull	Number of heifers	Days of lactation	Milk, kg	Butterfat		Qualification	Mycoplasma
				kg	%		
A(−)	53	288	4767	181.3	3.8	III	−
C(+)	31	293	4277	155.3	3.64	III	+
B(+)	28	273	4071	150.9	3.9	III	+
D(−)	62	282	4485	171.8	3.83		−
*E(+)							+
*F(+)							+

* No data were available.

Annual fluctuations in the reproductive-biological indices

The yearly fluctuations of the reproductive-biological indices for an uninfected bull were compared on heifers kept in the same farm. The data of this bull, A(−), are shown in Tables II, III and IV. The reproductive-biological indices of even the same bull show considerable variation, probably depending on the composition, management technology and feeding of the heifer stock in which the semen doses are being used. Similar variations were observed for bull D(−).

Comparison of reproductive-biological data for infected and uninfected bulls

Farms III and I. In farm III altogether 3 bulls were compared (Tables V and VI). The parameters of the two negative bulls were similar; the χ^2 test showed no significant differences. There were no differences between parameters of bulls A(−) and C(+) either. Between bulls D(−) and C(+) significant differences were found in the ratio of failure to success in respect of the whole stock

Table II

Distribution of heifers according to the number of insemination attempts in different years

Conception	Number of insemination attempts	A(—) Farm III		
		1981	1982	1983
+	1	179	71	48
	2	61	10	4
	3	25	2	—
	4	5	—	—
	5	1	—	—
	6	1	—	—
	Number of heifers	272	83	52
	Semen doses (*)	407 (56)	97 (8)	56 (1)
—	1	32	80	55
	2	31	15	17
	3	9	3	1
	4	5	—	—
	5	—	—	—
	Number of heifers	77	98	73
	Semen doses	141	119	92
	Total number of heifers	349	181	125
	Total number of semen doses	548	216	148

(*) In parentheses: the number of semen doses resulting in acyclia.

Table III

Reproductive-biological indices of bulls in annual distribution in farm III

Indices	A(—) Farm III		
	1981	1982	1983
(1) Ratio of not fertilizing to successfully fertilizing semen doses			
(a) in the conceived heifer group	0.50	0.17	0.08
(b) in the whole stock	1.01	1.60	1.85
(2) Ratio of heifers conceived after more than one insemination to those conceived at the first attempt	0.52	0.17	0.08
(3) Ratio of non-conceived to conceived heifers	0.28	1.18	1.40
(4) Ratio of semen doses not fertilizing at the first attempt to those successfully fertilizing at the first attempt			
(a) in the conceived heifer group	1.27	0.37	0.17
(b) in the whole stock	2.06	2.04	2.08
(5) Ratio of semen doses resulting in acyclia to successfully fertilizing semen doses in the conceived heifer group	0.16	0.09	0.02

Table IV

Annual comparison of reproductive-biological indices of bull A(—) (farm III) by the chi-squared (χ^2) test

Indices compared		P values*		
		1981	1982	1983
(1A) Number of not fertilizing and successfully fertilizing semen doses in the conceived heifer group	1981–1983	<0.001	<0.001	—
(1B) Number of not fertilizing and successfully fertilizing semen doses in the whole stock	1981–1983	<0.01	<0.01	—
(2) Number of heifers conceived after multiple inseminations and at the first attempt	1981–1983	<0.001	<0.001	—
(3) Number of non-conceived and conceived heifers	1981–1983	<0.001	<0.001	—
(4A) Number of semen doses failing to fertilize at the first attempt and successfully fertilizing at the first attempt in the conceived heifer group	1981–1983	<0.001	<0.001	—
(4B) Number of semen doses failing to fertilize at the first attempt and successfully fertilizing at the first attempt in the whole stock	1981–1983	—	—	—
(5) Number of semen doses resulting in acyclia and that of successfully fertilizing semen doses in the conceived heifer group	1981–1983	—	<0.05	—
(6) Distribution according to the number of insemination attempts	1981–1983	—	<0.001	—

* —: $P > 0.05$.

($P < 0.05$), in the ratio of non-conceived to conceived heifers ($P < 0.001$), and in the ratio of failures at first attempt to successful fertilization at the first attempt ($P < 0.05$).

In farm I only two bulls were available for comparison (Tables IV and V). In this case, most parameters of the mycoplasma-positive bull were inferior to those of the negative one. Only in the ratio of non-conceived to conceived heifers was the uninfected sire inferior ($P < 0.001$). There was a similarly significant ($P < 0.001$) difference in the ratio of semen doses failing to fertilize at the first attempt in the conceived heifer group, while no difference was found in the same ratio as regards the whole stock. There was no difference in the ratio of semen doses resulting in acyclia to those resulting in fertilization, whereas significant ($P < 0.05$) differences were found in the remaining indices including the distribution according to the number of insemination attempts.

Farms II and IV. In farm II the indices of 2, while in farm IV those of 3 bulls were examined. In farm II the indices of the mycoplasma-positive bull

Table V

Distribution of heifers according to the number of insemination attempts in farms III and I

Conception	Number of insemination attempts	Farm III			Farm I	
		A(—)	D(—)	C(+)	A(—)	B(+)
+	1	71	57	32	82	107
	2	10	6	7	21	45
	3	2	—	—	3	13
	4	—	—	—	2	2
	5	—	—	—	—	2
	6	—	—	—	—	—
	7	—	—	—	—	1
	Number of heifers	83	63	39	108	170
	Semen doses (*)	97 (8)	69 (5)	46 (5)	141 (17)	261 (25)
—	1	80	47	64	63	46
	2	15	10	6	16	12
	3	3	2	—	6	5
	4	—	—	—	3	1
	5	—	—	—	—	—
	Number of heifers	98	59	70	88	64
	Semen doses	119	73	76	125	89
	Total number of heifers	181	122	109	196	234
	Total number of semen doses	216	142	122	266	350

(*) In parentheses: the number of semen doses resulting in acyclia.

Table VI

Reproductive-biological indices of bulls in farms III and I

Indices	Farm III			Farm I	
	A(—)	D(—)	C(+)	A(—)	B(+)
(1) Ratio of not fertilizing to successfully fertilizing semen doses					
(a) in the conceived heifer group	0.17	0.10	0.18	0.31	0.54
(b) in the whole stock	0.60	1.25	2.13	1.46	1.06
(2) Ratio of heifers conceived after more than one insemination to those conceived at the first attempt	0.17	0.11	0.22	0.32	0.59
(3) Ratio of non-conceived to conceived heifers	1.18	0.94	1.79	0.81	0.38
(4) Ratio of semen doses failing to fertilize at the first attempt to those successfully fertilizing at the first attempt					
(a) in the conceived heifer group	0.37	0.21	0.44	0.72	1.44
(b) in the whole stock	2.04	1.49	2.81	2.24	2.27
(5) Ratio of semen doses resulting in acyclia to successfully fertilizing semen doses in the conceived heifer group	0.09	0.08	0.12	0.14	0.11

Table VII

Distribution of heifers according to the number of insemination attempts in farms II and IV

Conception	Number of insemination attempts	Farm II			Farm IV	
		C(+)	A(—)	D(—)	E(+)	F(+)
+	1	46	39	61	66	66
	2	19	4	4	23	22
	3	1	1	—	13	6
	4	7	—	—	2	—
	5	2	—	—	—	—
	6	1	—	—	—	—
	Number of heifers	76	44	65	104	94
	Semen doses (*)	131 (18)	50 (12)	69 (1)	159 (29)	128 (16)
—	1	14	17	32	34	48
	2	8	3	6	14	19
	3	1	1	—	9	3
	4	3	1	—	3	—
	5	2	—	—	—	—
	6	1	—	—	—	—
	Number of heifers	29	22	38	60	70
	Semen doses	61	30	44	101	95
	Total number of heifers	105	66	103	164	164
	Total number of semen doses	192	80	113	260	223

(*) In parentheses: the number of semen doses resulting in acyclia.

Table VIII

Reproductive-biological indices of bulls in farms II and IV

Indices	Farm II			Farm IV	
	C(+)	A(—)	D(—)	E(+)	F(+)
(1) Ratio of not fertilizing to successfully fertilizing semen doses					
(a) in the conceived heifer stock	0.72	0.14	0.06	0.53	0.36
(b) in the whole stock	1.53	0.82	0.74	1.50	1.37
(2) Ratio of heifers conceived after more than one insemination to those conceived at the first attempt	0.65	0.13	0.07	0.58	0.42
(3) Ratio of non-conceived to conceived heifers	0.38	0.50	0.58	0.58	0.74
(4) Ratio of semen doses not fertilizing at the first attempt to those successfully fertilizing at the first attempt					
(a) in the conceived heifer group	1.85	0.28	0.13	1.41	0.94
(b) in the whole stock	3.17	1.05	0.85	2.94	2.38
(5) Ratio of semen doses resulting in acyclia to successfully fertilizing semen doses in the conceived heifer group	0.16	0.32	0.01	0.22	0.14

Table IX

Comparison of reproductive-biological indices of bulls in farms II and IV by the chi-squared (χ^2) test

Indices compared	P values* Farm II		P values* Farm IV		
	C(+)	A(—)	D(—)	E(+)	F(+)
(1A) Number of not fertilizing and successfully fertilizing semen doses in the conceived heifer group		<0.01	<0.001 D—F	<0.01	<0.05
(1B) Number of not fertilizing and successfully fertilizing semen doses in the whole stock		<0.05	<0.001 D—F	<0.001	—
(2) Number of heifers conceived after multiple inseminations and at the first attempt		<0.01	<0.001 D—F	<0.001	<0.001
(3) Number of non-conceived and conceived heifers		—	— D—F	—	—
(4A) Number of semen doses failing to fertilize at the first attempt and successfully fertilizing at the first attempt in the conceived heifer group		<0.001	<0.001 D—F	<0.001	—
(4B) Number of semen doses failing to fertilize at the first attempt and successfully fertilizing at the first attempt in the whole stock		<0.001	<0.001 D—F	<0.001	—
(5) Number of semen doses resulting in acyclia and that of successfully fertilizing semen doses in the conceived heifer group		<0.01	<0.001 D—F	<0.01	—
(6) Distribution according to the number of insemination attempts		<0.001	<0.001 D—F	<0.001	—

* —: $P > 0.05$.

were considerably worse than those of the negative one. In farm IV the indices for the two mycoplasma-infected bulls were also significantly different from those for the negative bulls (Tables VII, VIII and IX).

Discussion

The successful culturing of mycoplasmas from semen samples deep-frozen in straws indicates that mycoplasmas are of considerable resistance to deep-freezing, a fact that has been unambiguously proved by laboratory experiments and data of the literature. Their viability is not influenced by the antibiotics present in the semen diluent.

Progeny-testing results of the bulls included in our study indicate that the milk and butterfat production of the heifers inseminated with mycoplasma-

positive semen and the number of progeny-tested offspring were lower than the same parameters of the heifers inseminated with the uninfected semen.

In the same farm, the semen taken from the very same bull resulted in varying conception rates in different years. This can be attributed to numerous causes the most important of which are the alterations in the composition of the heifer stock and in feeding. This fact emphasizes that the reproductive-biological indices of different sires can be compared only in the same heifer stock.

When comparing the same bulls in different farms, usually the indices of mycoplasma-infected bulls were significantly inferior as compared to those of the uninfected ones. This fact stresses the role of mycoplasmas, in accordance with data of the literature (Saed and Al-Aubaidi, 1983; Stalheim and Proctor, 1976; Hirth et al., 1970). Naturally, the weight of the individual parameters is different. The reproductive-biological data should be related to the conceived heifers, since the category of non-conceived ones includes a number of aetiological factors not necessarily indicative of bull semen quality (e.g. conceptivitiy of the heifers, technique of insemination, insufficient number of inseminations, etc.).

The indices applied by us are very close to the parameters generally used in animal breeding. Their use is justified by the easier implementation of the χ^2 test for the study of frequency distribution.

To a certain extent, our data render perceptible the magnitude of economic losses inflicted by mycoplasma infection. Although the numerical measurement of economic losses was beyond the scope of this study, we should like to indicate their magnitude by presenting the obtained data. For the mycoplasma-positive bulls, the ratio of not fertilizing to successfully fertilizing semen doses was usually lower than for the negative sires. Particularly large differences were observed in the ratio of semen doses not fertilizing to those successfully fertilizing already at the first attempt. Economic losses stem from increased semen demand and from the fact that in case of higher indices the heifers must be kept throughout several oestrous cycles. The ratio of cows conceived after more than one attempt to those conceived at first attempt is twice as high for the mycoplasma-infected bulls as for the non-infected sires. For the same reason, the ratio of semen doses resulting in acyclia also deserves attention. This index shows that economic losses arise not only from the surplus use-up of semen and from conception failures but also from a prolonged dioestrus (according to our data dioestrus can be as long as 3 months). In the latter case the heifers usually required uterine treatment. Thus, economic losses are increased by the extension of the service period and by treatment costs. In addition, the latter index suggests that the use of mycoplasma-containing semen for insemination initiates a pathological process in the uterus, leading to a prolongation of the dioestrus.

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ISOLATION OF A BOVINE ADENOVIRUS FROM FALLOW DEER *(DAMA DAMA)*

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A type 6 bovine adenovirus of subgroup II was isolated in calf testicle cell culture from the lungs and trachea of a succumbed fallow deer *(Dama dama)*, which had been kept in the Budapest Zoo, in a group of fallow deers showing mild respiratory symptoms. Convalescent sera of the surviving animals contained no antibodies to infectious bovine rhinotracheitis (IBR), respiratory syncytial (RS) and parainfluenza-3 (PI-3) viruses; however, antibodies to type 2 and 6 bovine adenoviruses were detectable. Based upon the characteristic gross pathological and histopathological changes, the demonstration of virus-neutralizing antibodies in the convalescent sera and the isolation of adenovirus it can be postulated that the primary cause of the respiratory disease was the type 6 bovine adenovirus.

Keywords. Bovine adenovirus, isolation, fallow deer *(Dama dama)*, respiratory disease.

Soon after the isolation and characterization of bovine adenoviruses from healthy cattle (Klein et al., 1959), these viruses were brought into causal relationship with the so-called pneumoenteritis of calves, a disease manifesting itself in respiratory and digestive symptoms (Bartha and Áldásy, 1964; Áldásy et al., 1964; Darbyshire et al., 1965). After several authors had reported the worldwide prevalence of adenovirus infection of cattle, Belák and Pálfi (1974) established the causative role of type 2 bovine adenovirus in the pneumoenteritis of sheep. More recently, Baber and Condy (1981) isolated type 3, 4 and 8 bovine adenoviruses from the nasal secretions of free-living, healthy Caffre buffaloes *(Syncerus caffer)*. The infection of other animal species has been demonstrated by results of serological surveys; e.g. Elazhary et al. (1981) demonstrated virus-neutralizing antibodies to bovine adenovirus in a caribou *(Rangifer tarandus caribou)* herd in Canada.

In the present paper isolation of a type 6 bovine adenovirus from the organs of a fallow deer died of respiratory disease is reported.

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Materials and methods

In the Budapest Zoological and Botanic Gardens four animals in a fallow deer group consisting of nine animals had to be immobilized for blood sampling. Immobilization was carried out using a combination of 1.5 mg/kg body mass xylazine and 1 mg/kg body mass ketamine shot into the animals with a gun; in addition, each animal received 10 to 20 mg atropine sulphate. During the veterinary intervention it was observed that the non-immobilized animals frequently coughed, particularly if they were forced to rapid movement or fleeing. The wet, not painful, coughing occurred 1 to 2 times per min. Of the immobilized animals an old stag also showed a strong, frequent coughing and swallowed the coughed-up phlegm. Three hours after the beginning of immobilization this animal stood up, and next morning he was found dead in the paddock. The remaining fallow deers continued to show mild coughing for a further 3 weeks; other clinical symptoms (inappetence, diarrhoea) were not observed. In the preceding months the animals exhibited no symptoms indicative of disease.

The old fallow deer stag having died of respiratory disease was subjected to detailed *gross pathological examination*. Pieces of the trachea, lungs, liver, kidneys, bronchi and peribronchial lymph nodes were fixed in 3.33 mol/l (10%) formalin, then embedded in paraffin. The sections were examined after staining with haemalaun and eosin.

Virological examination. Organ homogenates were prepared in a tenfold volume of Hanks's solution from parts of the pathologically-altered organs (lungs, trachea and the associated lymph nodes). The homogenates were processed in the usual way and were inoculated into tube cultures of primary or secondary calf kidney or calf testicle cell cultures. The cell cultures incubated at 37 °C in the stationary way were examined daily for cytopathic effects (CPE). From the cultures in which no CPE was observed up to the 7th day of incubation, five blind passages were made.

Virus identification. Cell cultures exhibiting CPE and those before passaging were fixed in methanol and stained with haemalaun and eosin. The isolate was examined for sensitivity to chloroform treatment. Type determination was performed by the virus-neutralization test using grade-II reference sera produced in conventional rabbits.

Serological examinations. No paired sera were available from the fallow deers showing respiratory symptoms, since the blood samples withdrawn from the 4 immobilized animals were taken and used for another purpose. Blood samples were taken only after 6 weeks from the 4 contacts of the fallow deer stag that died of respiratory disease; the blood samples were examined for antibodies to adenoviruses, IBR, RS and PI-3 viruses. Antibodies to adenoviruses, IBR and RS viruses were demonstrated by the virus-neutralization

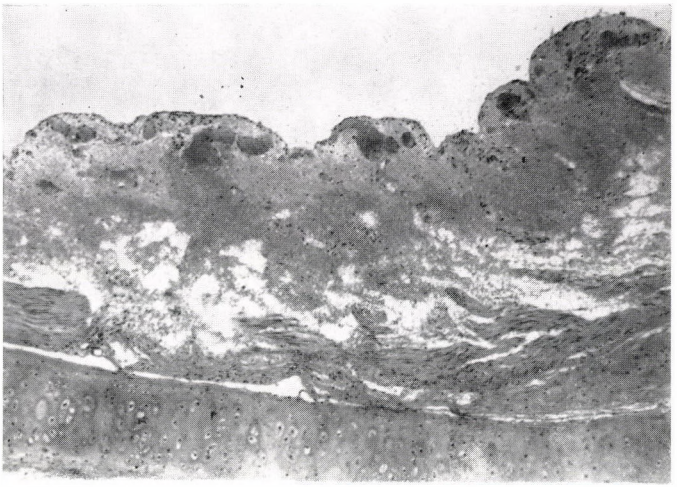

Fig. 1. The bronchial epithelial cells are detached, the mucous membrane contains extensive haemorrhages and serous-haemorrhagic infiltration. H. and E., × appr. 80

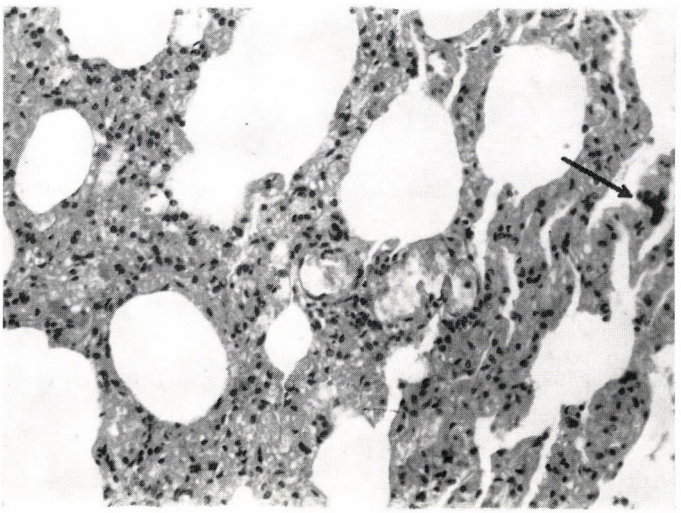

Fig. 2. Acute interstitial pneumonia; note the "smudge" cell (arrow) among the infiltrating cells. H. and E., × appr. 250

(VN), while those to PI-3 virus by the haemagglutination-inhibition (HI) test. In the VN test 100 $TCID_{50}$ virus, while for the HI test 4 HA units of the virus were used.

Results

At necropsy, the mucous membrane of the larynx, trachea and bronchi was markedly reddened and its surface was covered by a mucous coating containing fibrin shreds. The mucous membrane contained petechiae and linear haemorrhages. The apical lobes and the anterior one-third of the main lobes of the lungs were dark greyish-red in colour and of slightly compact consistency; a catarrhal-purulent material could be squeezed out onto the cut surface. In the marginal parts of the pathologically altered lung portions the cut surface was dark red and of meat-like consistency. In other parts of the lungs pathological changes indicative of acute pulmonary emphysema were observed. The peribronchial lymph nodes showed acute swelling.

By light-microscopic histopathological examination, severe hyperaemia of the mucous membrane of the trachea and bronchi, and extensive mucosal haemorrhages were seen. Detachment of epithelial cells occurred practically everywhere, and a mild lymphocytic infiltration was observed in the mucous membrane (Fig. 1.). In large areas of the lungs, the lumina of bronchioles and alveoli were filled with masses of neutrophilic granulocytes embedded in a substance that stained homogeneously pink with eosin; the alveolar epithelial cells were necrotic, and detached. In the marginal parts of areas showing the above pathological alterations no pathological contents were found in the lumina of alveoli; however, the intraalveolar septa became widened and were infiltrated by large numbers of histiocytes and lymphocytes. In the widened interstitial septa small numbers of gigantic so-called "smudge" cells with homogeneously dark-staining nuclei and of indefinite structure were observed (Fig. 2.).

In the peribronchial lymph nodes enlargement of germinal centres, hyperplasia of reticulum cells and mild infiltration by neutrophilic and eosinophilic granulocytes were seen. In other organs of the carcase no important pathological changes were found, neither by gross pathological nor by histopathological examination.

An adenovirus was isolated from the lung and the regional lymph node of the fallow deer stag died of respiratory disease. Based upon the results of the virus-neutralization test, the isolate designated DS/84 proved to be a type 6 bovine adenovirus of subgroup II. The CPE of the virus appeared first in the fourth passage, on the 5th day in inoculated calf testicle cell cultures. It was characterized by the appearance of diffusely-distributed, enlarged, refractile, rounded cells with sharp outlines in the monolayer cell cultures. The CPE occurred consistently during the passages; however, it did not extend to all cells

of the cultures by the 14th day. The virus titre reached 10^3. The isolate could not be propagated and caused no CPE in calf kidney cell cultures.

In stained preparations, eosinophilic-amphophilic intranuclear inclusion bodies of regular, round shape and sharp outline were seen, usually more than one per nucleus. The virus was resistant to chloroform treatment.

The serum samples from the 4 fallow deers contained no antibodies to IBR, RS and PI-3 viruses. On the other hand, the sera of all the four animals contained antibodies to the type 6 bovine adenovirus isolated, and two sera also to type 2 bovine adenovirus.

Discussion

Formerly adenoviruses had been regarded as strictly species-specific viruses, i.e. viruses that do not cross the borders between species. However, it was soon found that adenoviruses can occur also in other species; furthermore, they can cause a similar pathological condition and disease in these other species as in the species considered to be the characteristic host of the virus. The first, most classical example was infectious canine hepatitis (Rubarth's disease), a disease caused by adenovirus. Initially the disease had been considered to occur only in dogs; however, it soon turned out that foxes and bears may also be fatally affected.

Belák and Pálfi (1974) were the first to report the occurrence of bovine adenoviruses in species other than cattle. They established that the type 2 bovine adenovirus was the causative agent of an economically important respiratory and digestive disease of young sheep. Several authors proved the infection of sheep by serological examinations. Specific antibodies to bovine adenoviruses were found also in the caribou *(Rangifer tarandus caribou;* Elazhary et al., 1981). Recently, type 3, 4 and 8 bovine adenoviruses have been isolated from healthy free-living Caffre buffaloes *(Syncerus caffer;* Baber and Condy, 1981).

In the light of the above facts, the isolation of a bovine adenovirus from fallow deer is not surprising. It has to be established, however, whether the virus can be brought into causal relationship with the severe respiratory disease of fatal outcome. Although in the present case comparative serological examination of paired sera was not carried out, based upon the presence of adenoviral antibodies in, and absence of antibodies to IBR, RS and PI-3 viruses from, the four blood samples taken 6 weeks after the disease had passed off, the causative role of bovine adenoviruses in the respiratory disease of fallow deer can be postulated. This hypothesis is confirmed and supported by the successful virus isolation and the characteristic histopathological changes (acute tracheitis, acute interstitial pneumonia, presence of "smudge" cells in the lungs), which

are similar to those observed earlier in calves and lambs affected by adenoviral respiratory disease.

The respiratory disease passed off in a "benign" manner: in the group of fallow deers none of the animals showed clinical symptoms after 3 weeks. In our opinion, the death of the fallow deer stag exhibiting respiratory symptoms was due to the stress constituted by anaesthesia and immobilization.

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EXPERIMENTAL INFECTION OF LAYING HENS WITH AN ADENOVIRUS ISOLATED FROM DUCKS SHOWING EDS SYMPTOMS

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Forty ISA-Vedette laying hens were infected with the KT/80 virus isolated from ducks showing EDS symptoms, and another forty with adenovirus 127 of chicken origin. Both groups responded to infection similarly and showed an equal decrease of egg production. No difference was observed in egg quality and seroconversion between the groups.

Keywords. Egg drop syndrome, adenovirus, laying hen, duck, experimental infection.

Since the egg drop syndrome (EDS-76) of laying hens was first described in Western Europe (Van Eck et al., 1976) and its aetiology was elucidated (McFerran et al., 1978), the incidence and epizootiological characteristics of the disease have been studied in several countries. Although, according to McFerran's supposition, the duck is the natural host of the pathogen, an avian adenovirus of unique properties, most authors have adopted the view that a disease characterized by markedly dropped egg production and typical egg alterations develops only in chicken flocks, while in other bird species infection passes off symptomlessly.

This view has been supported also by reports on the successful demonstration of antibodies to EDS virus in healthy individuals of several other bird species; furthermore, similar virus strains have been isolated from healthy ducks as well (Baxendale, 1978; Schloer, 1980; Villegas et al., 1979; Zsák et al., 1982; Bartha et al., 1982). Studies on the pathogenicity of a haemagglutinating avian adenovirus isolated in the United States from a healthy duck revealed that no decrease of egg production occurred in the susceptible laying hens experimentally infected with this virus (Brugh et al., 1984).

Recently a case of dropped egg production has been observed in a susceptible duck flock (Bartha, 1984). Since EDS adenovirus was supposed to play a role in the aetiology of this condition, the pathogenicity of the KT/80 virus strain isolated from this duck flock was examined and compared with the pathogenicity of the adenovirus reference strain 127 in susceptible laying hens.

Eighty individually marked, 19 weeks old ISA-Vedette pullets were placed in two isolated rooms. No EDS had occurred in the flock of origin and all

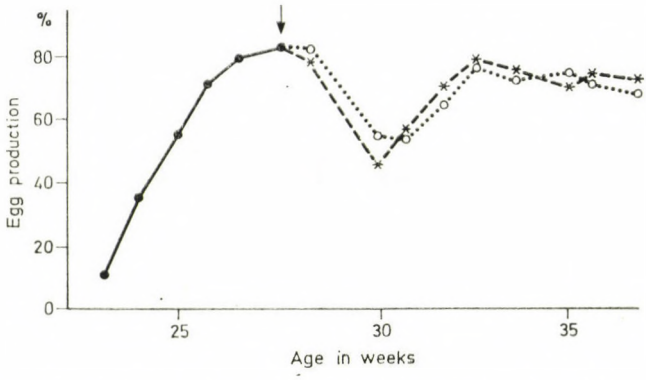

Fig. 1. Egg production of hens infected with adenovirus 127 (x·····x) or with duck adenovirus KT/80 (0 · · · · 0)

layers had been seronegative. The pullets started to lay eggs at the age of 22 weeks, and egg production reached the peak in the 27th week (80%). In the 28th week, the hens were infected orally with 0.5 ml duck embryo allantoic fluid containing a virus quantity of 10^6 median embryo infective dose (EID_{50}). Forty layers were infected with adenovirus 127, while the other forty with the KT/80 virus of duck origin.

Egg production was recorded and egg quality was assessed daily. One week after infection hens of both groups started to lay thin-shelled, soft-shelled or shell-less eggs, and continued to lay such eggs for three weeks thereafter. Egg production is shown in Fig. 1.

For the demonstration of haemagglutination-inhibition (HI) antibodies, the microtiter method was used, the HA antigen consisted of adenovirus 127.

In the present experiment the susceptible laying hens infected with the KT/80 virus strain isolated from ducks and with adenovirus 127 of chicken origin responded to infection similarly. An equal decrease of egg production

Table I

HI antibody titres of hens before and after exposure to EDS avian adenovirus isolated from duck and chicken

Number of pullets	Infected with	Day of sampling after challenge	Number of sera with reciprocal HI antibody titre of						
			8	8	16	32	64	128	256
40	duck adenovirus KT/80	0	40						
		14			1	1	4	10	24
		35		1	2	3	7	19	8
40	chicken (fowl) adenovirus 127	0	40						
		14			1	4	2	9	24
		35			2	4	4	20	10

was seen in both groups, no difference was observed in egg quality, and seroconversion was also similar in the two groups (Table I).

These results lack conformity with those of Brugh et al. (1984), who failed to produce in laying hens dropped egg production with the haemagglutinating adenovirus isolated from a healthy duck flock. It is suggested that these contradictory results are due to genetic differences in pathogenicity between the virus strains used.

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A BOVINE HAEMAGGLUTININ OF *BORDETELLA BRONCHISEPTICA* RESPONSIBLE FOR ADHERENCE

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(Received January 18, 1985)

Bordetella bronchiseptica strains freshly isolated from swine herds with atrophic rhinitis (AR) showed a similar haemagglutination pattern including agglutination of calf erythrocytes (calf-positive strains). After several passages these strains lost the ability to agglutinate calf and horse red blood cells (RBCs) but retained haemagglutinating activity on RBCs from other animal species (calf-negative strains). Phase III strains failed to agglutinate calf RBCs; neither did they agglutinate sheep erythrocytes. In the in vitro experiment, calf-positive strains attached to the cilia of epithelial cells, whereas calf-negative variants failed to do so. It is postulated that this new type of haemagglutinin in *B. bronchiseptica* strains may act as an adhesin and as a virulence factor in the pathogenesis of AR in swine.

Keywords. *Bordetella bronchiseptica*, haemagglutination spectrum, adherence, virulence factor.

The ability of *Bordetella* species to agglutinate erythrocytes from various mammals and fowl was first described by Keogh et al. (1947). This observation was confirmed by Goodnow (1980). The haemagglutinin (HA) produced by *Bordetella pertussis* has been extensively investigated over the past 20 years and as a result of these works it is known that this species elaborates two distinct HAs (Arai and Sato, 1976; Morse and Morse, 1976; Irons and MacLennan, 1979; Arai and Munoz, 1979). One of them is associated with the fimbriae, is nontoxic, seems to be protective, and has filamentous structure, therefore, it is called fimbrial haemagglutinin (F-HA). The other haemagglutinin (lymphocytosis-promoting factor, LPF-HA) is associated with a toxic protein appearing as spherical structures in the electron microscope, has a relatively low haemagglutinating activity, produces a variety of biological effects including leukocytosis, histamine hypersensitivity, and lethality. This substance is also referred to as pertussigen (Munoz and Bergman, 1977). The recent separation and purification of the HAs from *B. pertussis* (Sato et al., 1983) may contribute to a better understanding of the role of these two HAs in the pathogenesis and immunoprophylaxis of whooping cough. *Bordetella bronchiseptica*, one of the supposed causative agents of atrophic rhinitis (AR) of swine, has many similarities to *B. pertussis*. This bacterium species also agglutinates

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the erythrocytes of various animals, but LPF-HA has been found only in cultures of *B. pertussis* and not in those of *B. bronchiseptica* (Pittman, 1979). Sato et al. (1979) have proposed that the F-HA of *B. pertussis* is derived from the fimbriae on the bacterial surface, and a fimbria-preparation from a strain of *B. bronchiseptica* has been shown to agglutinate chicken erythrocytes (Blom et al., 1983).

It is well known that fimbriae (pili) play an important role in the bacterial adherence to the host cells and thereby in the colonization of mucosal surfaces, which is generally the first step of infection (Isaacson, 1983; Ofek and Beachey, 1980). There is a close correlation between piliation and haemagglutination, hence the simple and inexpensive haemagglutination test has been widely used to explore the fimbria-producing and adhering capability of strains of *Escherichia coli* (Semjén and Magyar, 1983). Although haemagglutination by *B. bronchiseptica* has been recognized by several authors, our knowledge is rather scanty in this field. The single thorough investigation by Bemis and Plotkin (1982) on the haemagglutination of *B. bronchiseptica* did not make clear the role of HAs in the attachment of *B. bronchiseptica* to respiratory cells. Yokomizo and Shimizu (1979) described that phase I *B. bronchiseptica* attached to the cilia of swine nasal epithelial cells, whereas phase III variants did not. More recently Tuomanen et al. (1983) demonstrated differences in the adherence of *Bordetella* species to ciliated cells from human beings and those from various laboratory animals. In preliminary publications (Semjén et al., 1983; Magyar et al., 1983) we reported that some *B. bronchiseptica* strains of porcine origin agglutinated calf red blood cells (RBCs) and adhered well to ciliated epithelial cells from pigs. A detailed account on the topic is presented in this paper.

Materials and methods

Bacterial strains

A total of 16 *B. bronchiseptica* strains were used. Freshly-isolated strains originated from nasal swabs of pigs at different farms with mild or moderate clinical AR. The isolation was made on MacConkey agar containing penicillin (20 mg/l) and nitrofurantoin (20 mg/l), and/or on G20G medium described by Smith and Baskerville (1979). The identity of *B. bronchiseptica* was confirmed by biochemical tests and by slide agglutination test with antiserum prepared in rabbits by intravenous injections of *B. bronchiseptica*. Each strain, investigated in this work, represented one farm and was designated with the initials of the farm's name. All freshly-isolated strains were phase I organisms, except one (31Ch), which was isolated from a pig of a herd without clinical symptoms of AR. This strain and a type strain of *B. bronchiseptica* ATCC (received from

the American Type Culture Collection, Rockville, Maryland, U. S. A.) showed phase III colony morphology. The freshly-isolated strains were maintained on Dorset's egg yolk slants throughout the experiment. Subcultures, obtained by passaging several times on MacConkey agar, were designated with the prefix "p" and the number of passages.

Haemagglutination test

The strains were cultivated for 24 or 48 h on Blood Agar Base (BAB) medium (Oxoid) with or without 5 per cent sheep blood at 37 °C and on BAB medium with 5 per cent sheep blood at 20 °C. In general, haemagglutination tests were performed by slide agglutination using 5 per cent RBC suspensions in phosphate-buffered saline (PBS), pH 7.2, from calf, horse, sheep, guinea-pig, pig, dog, rabbit, chicken, and type-A human blood. A portion of bacterial growth was picked off with a straight inoculating needle and mixed with a drop (0.025 ml) of RBC suspension from the appropriate species on a glass slide at room temperature. After mixing by rotation of the slide for 2 min, the results were recorded as follows: a ++++ reaction was the quick and complete agglutination of all RBCs; lesser degrees of haemagglutination were recorded as +++, ++, + or negative. For investigating the effect of heat and formalin on haemagglutination, whole bacterial cell suspensions from 24-h blood agar cultures were prepared in PBS and adjusted to contain approximately 10^9 colony-forming units (CFU) per ml. These suspensions were treated with formalin (0.5%) at 37 °C overnight or heated to 60 °C in waterbath for 1 h. One drop of treated bacterial suspension was mixed with an equal volume of RBC suspension on slide and the reaction was scored as mentioned above.

For determining haemagglutination titre, the test described by Bemis and Plotkin (1982) was used in some cases.

Adherence test

The bacteria were harvested after incubation at 37 °C for 24 h on 5 per cent sheep blood agar, suspended in PBS, and standardized spectrophotometrically to contain about 10^9 CFU/ml. Ciliated nasal epithelial cells were collected by scraping the mucosa of the nasal conchae of piglets with a scalpel. The cells were suspended and washed 3 times in PBS and adjusted to contain approximately 10^6 cells per ml. With all strains, one ml bacterial suspension was mixed to one ml epithelial cell suspension, incubated at 37 °C for 20 min, and subsequently the mixtures were washed 3 times. After each washing the suspensions were centrifuged at 500 rpm for 10 min. The last sediment was examined under a phase-contrast microscope. The bacterial cells attached to 20 epithelial cells were counted, and the mean and the standard error of the mean were calculated by Student's *t* test for all strains.

Results

Freshly-isolated strains behaved in the haemagglutination test uniformly: they agglutinated RBCs of all the species tested, the intensity of agglutination, however, showed species-to-species variations. For example, agglutination of calf, horse, dog, and sheep erythrocytes was stronger than that obtained with human A, rabbit and guinea-pig RBCs, and there was very slight agglutination with chicken RBCs. The haemagglutination was not influenced by the duration (24 or 48 h) of incubation. The most remarkable finding was that most strains which were passaged several times and retained their S colony morphology, failed to agglutinate calf and horse erythrocytes (calf-negative strains), while their behaviour against RBCs from other species remained unaltered.

The haemagglutinating spectrum of strain BAKp36 was the same as before passages, indicating that in this case 36 transfers were not enough to cause any alteration in haemagglutinating activity. The geometric means of titres obtained with calf and sheep blood cells in tests repeated 4 to 6 times were between 22.6 and 64 for the different strains. Comparing the geometric means of haemagglutination titres, no significant differences were found between calf and sheep RBCs in the degree of agglutination, and the calf-negative variants gave nearly as strong agglutination with sheep RBCs as did the calf-positive parent strains.

The two phase-III strains (ATCC and 31Ch) did not agglutinate calf, horse, and sheep RBCs. The characteristic haemagglutination pattern of the *B. bronchiseptica* strains is summarized in Table I.

Table I

Haemagglutination patterns of *B. bronchiseptica* strains

Strains	Agglutination with RBCs from				
	calf	horse	sheep	pig	guinea-pig
MH13	++++	++++	+++	+++	+++
DK2	++++	++++	++	+++	++
FGT	++++	++++	+++	+++	+++
B10	++++	++++	+++	+++	+++
CE	++++	++++	++	+++	+++
CF	++++	++++	+++	+++	+++
FG5	++++	++++	+++	+++	+++
BÁB	++++	++++	+++	+++	+++
BAK	++++	++++	+++	+++	++
ATCC	—	—	—	+++	+++
31Ch	—	—	—	++	+++
CEp27	—	—	++	+++	+++
CFp61	—	—	+++	+++	+++
FG5p35	—	—	+++	+++	++
BÁBp37	—	—	+++	+++	+++
BAKp36	++++	++++	+++	+++	++

Table II shows the effect of formalin and heat treatment and of certain conditions of culturing on the haemagglutination of calf-positive and negative strains. When calf-positive and negative strains were grown at 20 °C or on BAB medium without blood, no agglutination of RBCs from calf occurred but the reaction with RBCs from other animal species remained unchanged. Heat treatment at 60 °C exerted a reverse influence on haemagglutination. Formalin treatment of calf-positive strains totally abolished all the haemagglutinating activity. Mannose, glucose, galactose, maltose, and lactose in a concentration of 0.5 per cent, N-acetyl-glucosamine and α-methyl-mannoside (0.25 M) had no influence on the haemagglutination of calf RBCs.

In the in vitro adherence experiments all the freshly-isolated calf-positive *B. bronchiseptica* strains adhered well to the cilia of ciliated epithelial cells of the nasal mucosa (Table III). On the other hand, neither the ATCC and 31Ch strains of R colony type nor the subcultures that have lost the ability to agglutinate calf and horse RBCs adhered to epithelial cells (except for accidental adherence of one or two bacterial cells on a few epithelial cells).

Discussion

The haemagglutinating ability of *B. bronchiseptica* and *B. pertussis* has been known for a long time. Recently our knowledge concerning *B. pertussis* HAs has widened. However, relatively few, mostly tangential, data are avail-

Table II

Effect of cultural conditions and treatment on haemagglutination by calf-positive and calf-negative strains

Cultural conditions and treatment	Phenotype[a]	Agglutination with RBCs from		
		calf	pig	guinea-pig
Live suspensions from BAB[b] with 5% sheep blood, cultivation at 37 °C	CP	+	+	+
	CN	—	+	+
Live suspension, cultivation at 20 °C	CP	—	+	+
	CN	—	+	+
Formalin treatment	CP	—	—	—
	CN	—	+	+
Heat treatment	CP	+	—	—
	CN	—	—	—
Live suspensions from BAB without blood	CP	—	+	+
	CN	—	+	+

[a] CP = calf-positive strains, CN = calf-negative strains
[b] BAB = blood agar base (Oxoid)

Table III

Comparison of haemagglutinating and adhesive properties of *B. bronchiseptica*

Strains	Number of bacteria adhered to epithelial cells (mean ± SEM[1])	Agglutination with RBCs from calf
MH13	29.21 (±1.42)	++++
DK2	17.05 (±1.23)	++++
FGT	15.36 (±1.19)	++++
B10	15.00 (±0.93)	++++
CE	14.80 (±1.29)	++++
CF	17.05 (±1.76)	++++
FG5	20.00 (±1.08)	++++
BÁB	18.70 (±1.06)	++++
BAK	17.10 (±1.20)	++++
ATCC	0.35 (±0.18)	—
31Ch	0.85 (±0.33)	—
CEp27	0.38 (±0.16)	—
CFp61	0.10 (±0.10)	—
FG5p35	0.65 (±0.23)	—
BÁBp37	0.65 (±0.28)	—
BAKp36	14.35 (±1.02)	++++

[1] Standard error of the mean

able on the haemagglutination of *B. bronchiseptica* strains playing an aetiological role in AR, and neither the properties of the HAs nor their role and relationship with adherence are known sufficiently. Joubert et al. (1960) reported that a porcine strain agglutinated sheep and human type O RBCs but not horse RBCs. Kang et al. (1970) observed that only one of 12 *B. bronchiseptica* strains from pigs agglutinated horse RBCs. Contrarily, Bemis and Plotkin (1982), who were the first to study the haemagglutination spectrum and relative potency of *B. bronchiseptica* strains isolated from pigs and dogs, found that 10 of 11 porcine strains (91%) gave positive reaction with horse, sheep, dog, pig, and guinea-pig RBCs. These authors did not use calf blood; neither did they study the effect of serial passages.

In the present studies, the strains freshly isolated from herds affected with AR agglutinated the RBCs of all species studied, including calf. However, after different numbers of passages on MacConkey agar plates all subcultures but one lost their ability to agglutinate calf and horse RBCs, and did not regain this ability even after multiple subcultures on blood agar. As the agglutination of horse and calf RBCs ran parallel in our hand, we supposed that the reaction with RBCs from these two species was elicited by the same mechanism. Strains agglutinating bovine RBCs were designated calf-positive strains, while the substance responsible for eliciting agglutination was called bovine HA. No phenotypic manifestation of calf-positivity was observed at low temperature, on BAB or MacConkey agar cultures without blood. This is

not surprising since in bacteriology there are several examples that under adverse conditions certain energy-intensive, non-essential functions become repressed. At temperatures below 20 °C *E. coli* fails to produce K88 fimbria; thus, such cultures do not agglutinate sheep RBCs (Stirm et al., 1967).

After formalin treatment, calf-positive strains agglutinated the RBCs of none of the species studied. After heat treatment, only the bovine HA remained intact. Contrarily, formalin had no effect on calf-negative strains, but heat treatment deprived them from their agglutinating ability. The results obtained for the calf-positive strains are in sharp contrast with those reported by Bemis and Plotkin (1982). However, the results are not comparable, since the latter authors tested the HA of only one pair of strains isolated from dogs (110 H: piliated and 110 NH: non-piliated) against sheep RBCs.

Phase III ATCC and 31Ch strains did not agglutinate calf, horse, and sheep RBCs. Éliás et al. (1982) made similar observations. The fact that also phase III strains agglutinate the RBCs of pigs, dogs, and some other species allows us to conclude that *B. bronchiseptica* strains possess more than one HA. The haemagglutination spectrum of the strains is influenced also by the culture medium and conditions of culturing. This explains the contradictory result reported earlier.

The most important conclusion of our investigations is that we have revealed a relationship between the calf-HA of the strains and their adherence to ciliated epithelial cells. Based upon the present studies we may postulate that calf-HA functions also as an adhesin and contributes to the virulence of *B. bronchiseptica*.

This postulation can serve as a working hypothesis for further investigations needed to obtain conclusive evidence.

Acknowledgement

The authors are indebted to Mrs. Éva Blaskó for her skilful assistance.

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INVESTIGATION OF ADHESIVE AND NONADHESIVE *BORDETELLA BRONCHISEPTICA* STRAINS IN A SUCKLING-MOUSE MODEL

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Bordetella bronchiseptica strains isolated from pigs affected with atrophic rhinitis (AR), each agglutinating calf red blood cells (calf-positive strains), and calf-negative variants derived from the fresh isolates by serial passages were studied in a suckling-mouse model.

Calf-positive strains produced rhinitis of varying severity, in some instances accompanied by a pronounced turbinate atrophy. On the other hand, calf-negative variants either failed to induce pathological changes or caused only mild inflammation.

Keywords. *Bordetella bronchiseptica*, virulence, mouse model.

Atrophic rhinitis (AR) of swine, a disease causing heavy economic losses, has become the subject of more and more intensive research. However, its aetiology has still remained a question of much controversy.

For a long time, *Bordetella bronchiseptica* had been regarded as the primary aetiological agent of AR (Shimizu et al., 1971; Kemeny, 1972), but recently the importance of *Pasteurella multocida* has come to the fore (de Jong et al., 1980), although certain Japanese authors still are of the opposite opinion (Sawata et al., 1984). The most probable explanation is that toxin-producing *P. multocida* strains are responsible for inducing severe clinical symptoms, but virulent *B. bronchiseptica* strains also play an important role in the development of initial lesions. This hypothesis was supported by the investigations of Pedersen and Barfod (1981) and Rutter (1983), who, by infecting pigs with *B. bronchiseptica* followed by their inoculation with toxin-producing *P. multocida*, were able to produce symptoms corresponding to those of natural AR. According to recent investigations of Pedersen and Elling (1984), the effect of *B. bronchiseptica* can be simulated by chemical irritation with mild acetic acid. Under field conditions, however, it seems more probable that *B. bronchiseptica*, a bacterium causing respiratory disease in several animal species and characterized by a high affinity to the mucous membrane of the respiratory tract, is involved in initiating the disease process.

Based upon all these facts, investigations into the biological properties of *B. bronchiseptica* and into factors determining the virulence of the strains

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are essential. The suckling-mouse test described by Sawata and Kume (1982) seems to be an in vivo model applicable for this purpose. These authors found that while infection of young mice with phase I *B. bronchiseptica* organisms resulted in turbinate atrophy, the phase III strain failed to produce such lesions.

In the accompanying paper (Semjén and Magyar, 1985; see pp. 129—136 in the present volume) we report that *B. bronchiseptica* possessed at least two distinct haemagglutinins (HAs), of which the bovine HA was responsible for adherence to ciliated nasal epithelial cells. In the present work the effect of calf-positive and calf-negative strains on intranasally-inoculated suckling mice was investigated.

Materials and methods

Experimental animals

Nine litters, each consisting of 10 suckling mice of strain CFLP (LATI, Gödöllő), were used. All animals within a litter were inoculated with the same *B. bronchiseptica* strain on the second day of life. Nne litter served as untreated control.

B. bronchiseptica strains

Calf-positive *B. bronchiseptica* strains (CF, FG5, CE, FGT), calf-negative variants (CFp61, FG5p35, CEp27) and a phase III strain (31Ch) were used (for their description, see the accompanying paper; Semjén and Magyar, 1985). In brief, calf-positive strains were isolated from cases of clinical AR, whereas calf-negative variants were derived from the fresh isolates by serial passages on MacConkey agar. Strains CF, FG5, CE and FGT agglutinated calf red blood cells (RBCs) and adhered to isolated nasal epithelial cells, while strains CFp61, FG5p35, CEp27 and 31Ch had lost their calf-positivity and adhesive property.

Inoculation of suckling mice

B. bronchiseptica strains incubated at 37 °C for 24 h on 5% sheep blood agar plates were suspended in phosphate-buffered saline (PBS). The concentration of the suspensions was adjusted to approximately 10^8 colony-forming units (CFU) per ml by photometry. The infective dose was 3 μl, containing approximately 3.3×10^5 CFU. The mice were inoculated intranasally, using a Hamilton precision syringe.

Light-microscopic examination

Mice were killed 18 days post-inoculation (PI). The nasal region was fixed in 10% formalin, processed, embedded in paraffin and sectioned according to the routine procedure. The sections were stained with haematoxylin and eosin.

Results

All mice survived the infection. The severity of the lesions caused by the different *B. bronchiseptica* strains is shown in Table I. The most pronounced differences occurred among mice infected with strain CF and those inoculated with strain CFp61; therefore, these are described in detail. The mice inoculated with *B. bronchiseptica* strain CF showed differences in growth rate. The severity of lesions was correlated with the degree of retardation in growth. Of the 10 mice infected with strain CF, 4 showed severe, 3 moderate, and 3 mild histopathological changes.

Contrarily, the mice inoculated with *B. bronchiseptica* subculture CFp61 exhibited no lesions and their histopathological picture corresponded to that of the untreated controls (Figs 1, 3 and 5). The mice infected with strain CF developed lesions characterized by inflammation of the nasal mucosa and turbinate atrophy (Fig. 2). The olfactory epithelium was infiltrated by inflammatory cells, primarily neutrophil granulocytes (Fig. 4). The respiratory epithelial cells underwent degeneration and lost their cilia. Polymorphonuclear leucocytes were present in the epithelium (Fig. 6), sometimes forming microabscesses (Fig. 7). The epithelium was hyperplastic in some places, particularly on the scrolls of the dorsal turbinate (Fig. 8). In the nasal mucosa of mice showing more severe lesions, the lamina propria was infiltrated by neutrophils and mononuclear inflammatory cells (Fig. 9). Large quantities of inflammatory exudate, containing numerous polymorphonuclear and fewer mononuclear cells, accumulated in the nasal cavity (Figs 4 and 8).

Table I

Histopathological lesions in the nasal cavity of suckling mice inoculated intranasally with *Bordetella bronchiseptica* strains

Strain	Nasal lesion scores□			
	0	1	2	3
CF	0	3	3	4*
CFp61	10	0	0	0
FG5	0	4	5	1
FG5p35	6	4	0	0
CE	4	3	3	0
CEp27	7	3	0	0
FGT	0	4	4	2
31Ch	8	2	0	0

□ 0 = no lesion
1 = mild or moderate inflammation without any sign of atrophy
2 = moderate or severe inflammation with initial signs of atrophy
3 = apparent atrophy
* Each experimental group included 10 mice.

In mice infected with the other *B. bronchiseptica* strains, pronounced atrophy of turbinates was observed only occasionally. These strains produced mostly inflammatory changes of varying severity. Hardly more than a half of the animals inoculated with strain CE developed histopathological lesions, while in the remaining mice no lesions occurred at all.

Discussion

B. bronchiseptica is known to be the cause of respiratory diseases in numerous animal species. In mice, hitherto only Sawata and Kume (1982) were able to produce turbinate atrophy by experimental infection with *B. bronchiseptica*. In the present work, we have succeeded in producing the disease in mice; however, as regards the infective dose, we observed considerable differences from that reported by Sawata and Kume (1982). In our experiment, the 20 CFU/mouse dose used by these authors failed to give rise to any lesion with either of the strains applied. Based upon the results of several experiments, 3.3×10^5 CFU/mouse was selected as the infective dose of choice. This is in accordance with the results reported by Martineau et al. (1982), who studied in gnotobiotic piglets the infection pressure needed to induce the disease with *B. bronchiseptica*. According to them, the minimum infective dose was 3×10^5 CFU/ml when 0.5 ml of the bacterial suspension was inoculated into each nostril. None of the typical lesions developed at lower infective doses.

The differences found in the infective dose might be attributed to the fact that the strain used by Sawata and Kume (1982) was much more virulent than those applied in our experiment, or perhaps our experimental mice were more resistant to infection. Experimental infection with the calf-positive *B. bronchiseptica* strains used by us resulted in turbinate atrophy only in part of the mice; in most instances they produced only rhinitis of varying severity, without signs or with only initial signs, of atrophy. Strain CE failed to cause any lesion in four mice. On the other hand, in some cases mild inflammatory changes developed also after infection with strains that had lost their adhesive property. This, at least with the relatively high number of bacteria used for infection, indicates that factors other than adhesive ability might play an important role in inducing the process. In any case, further studies are needed to arrive at a better understanding of this problem.

Sawata and Kume (1982) reported that approximately 20% of the mice infected with phase I *B. bronchiseptica* microorganisms succumbed to pneumonia. On the other hand, in our experiment all the mice survived the infection, the only pathological finding being that mice infected with *B. bronchiseptica* strains possessing adhesive property, showed differences in growth rate. Retarded growth was in positive correlation with the severity of histopathological lesions. The general picture of the nasal cavity was identical with that found

by the above-cited authors. The histopathological lesions observed in the mucous membrane were similar to those reported for the experimental infection of piglets with *B. bronchiseptica* (Miniats and Johnson, 1980; etc.).

Hanada et al. (1979) established that a cell-free sonicated extract of toxin-producing *B. bronchiseptica* strain gave rise to development of AR in piglets. This fact indicates that the heat-labile exotoxin is responsible for turbinate atrophy in pigs.

The preliminary results of our current investigations suggest that our calf-positive *B. bonchiseptica* strains and calf-negative variants differ not only in adhesive ability but also in toxin-producing capacity (our unpublished data). This fact indicates that toxin-producing ability also decreases during serial passages. Further studies are needed to determine the degree to which the properties lost by the strains (adhesive ability and toxin-producing capacity) contribute to virulence.

In summary, for the practical applicability of the mouse model it is essential *(i)* to study the virulence factors of *B. bronchiseptica* by other tests, and *(ii)* to obtain a deeper understanding of differences in the susceptibility of mice to *B. bronchiseptica* infection.

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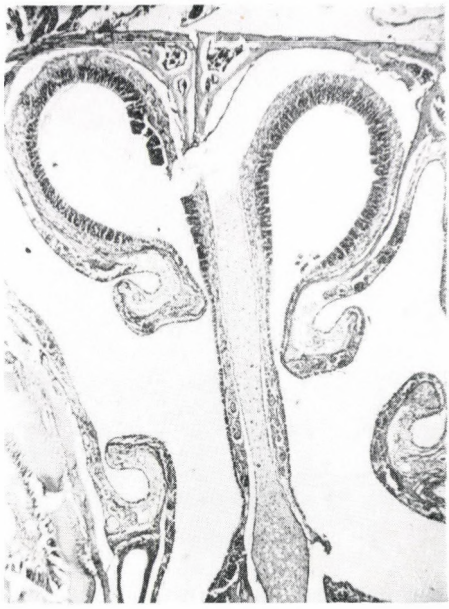

Fig. 1. Transversal section of the nasal cavity from a mouse infected with *B. bronchiseptica* strain CFp61. No pathological changes are seen. H.–E. stain, ×16

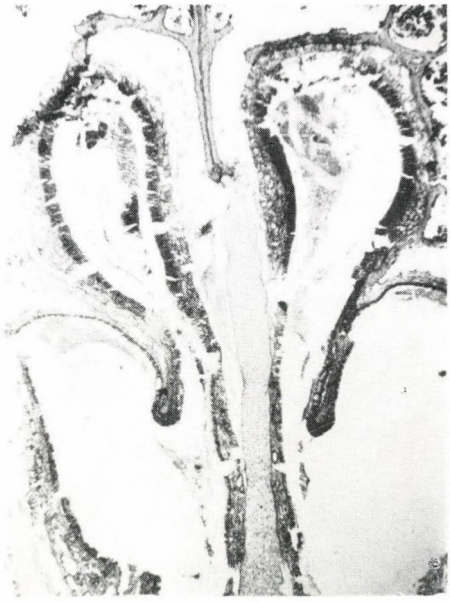

Fig. 2. Transversal section of the nasal cavity from a mouse infected with *B. bronchiseptica* strain CF. Atrophy of the dorsal turbinate. H.–E., ×16

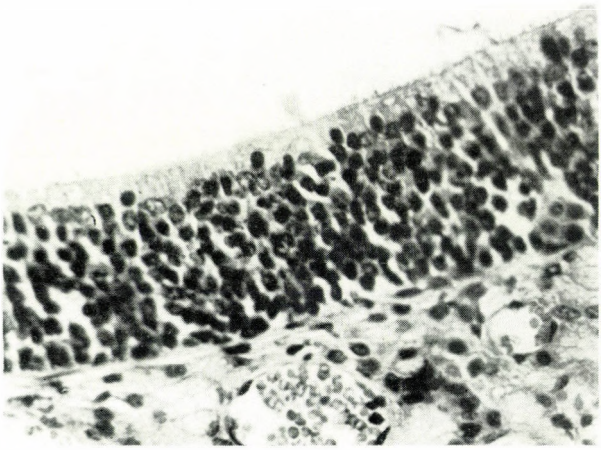

Fig. 3. Olfactory epithelium from a mouse infected with *B. bronchiseptica* strain CFp61. No pathological changes are seen. H.–E., ×140

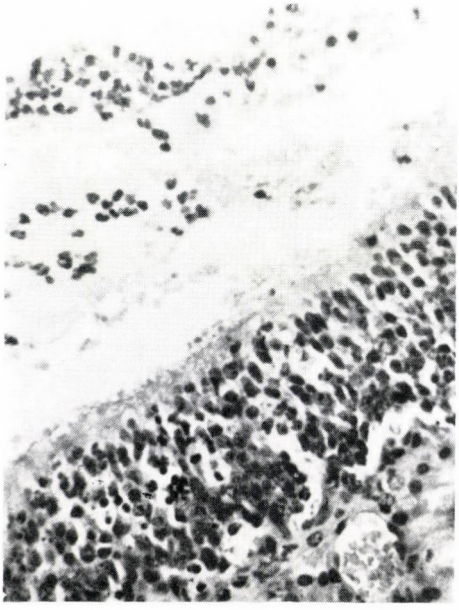

Fig. 4. Olfactory epithelium from a mouse infected with *B. bronchiseptica* strain CF. The epithelium is infiltrated by neutrophil granulocytes; the nasal cavity contains inflammatory exudate and cells. H.–E., ×140

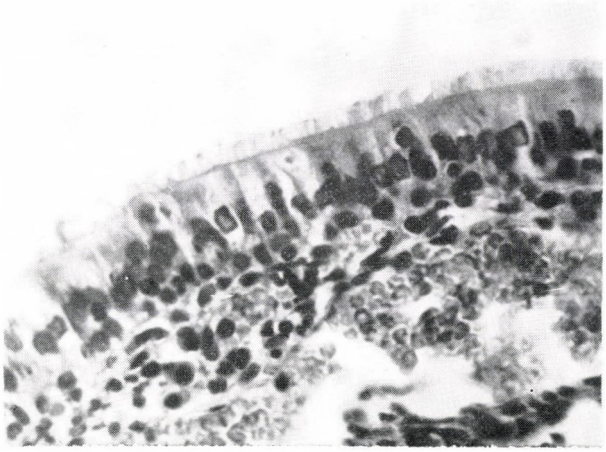

Fig. 5. Nasal respiratory epithelium from a mouse infected with *B. bronchiseptica* strain CFp61. No pathological changes are seen. H.–E., ×140

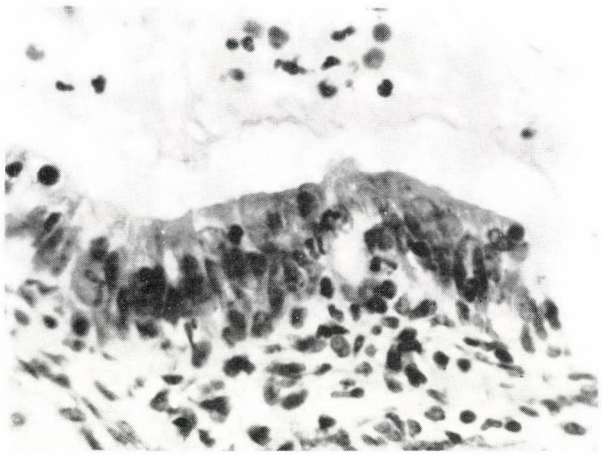

Fig. 6. Nasal respiratory epithelium from a mouse infected with *B. bronchiseptica* strain CF. Note the degeneration of epithelial cells, loss of cilia, and accumulation in the nasal cavity of inflammatory exudate containing mainly polymorphonuclear cells. H.–E., ×140

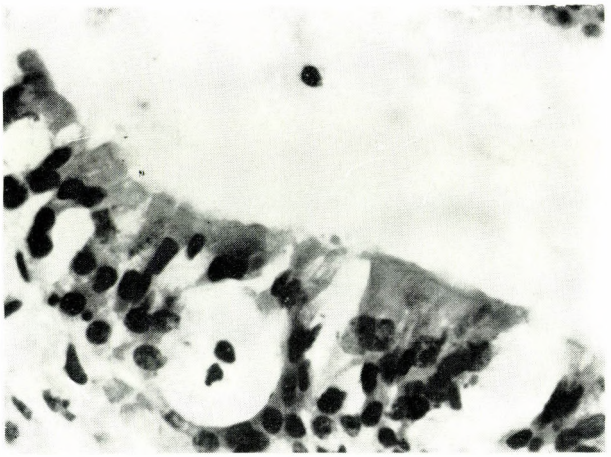

Fig. 7. Nasal respiratory epithelium from a mouse infected with *B. bronchiseptica* strain CF. A microabscess is seen in the epithelium devoid of cilia. H.–E., ×140

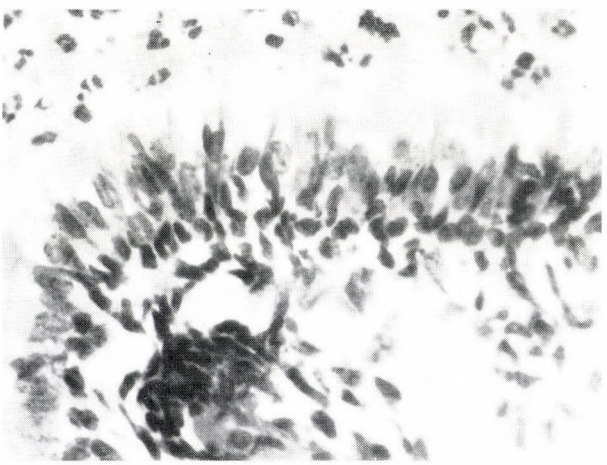

Fig. 8. Nasal respiratory epithelium from a mouse infected with *B. bronchiseptica* strain CF. Epithelial hyperplasia on the scrolls of the dorsal turbinate. Inflammatory cells in the nasal cavity. H.–E., ×140

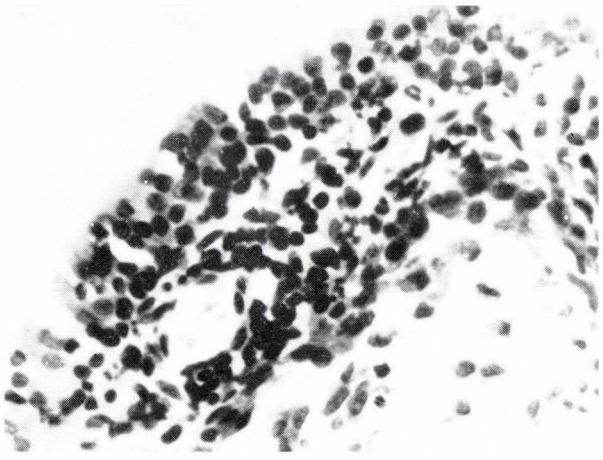

Fig. 9. Nasal respiratory epithelium from a mouse infected with *B. bronchiseptica* strain CF. The epithelium and the lamina propria are infiltrated by neutrophils and mononuclear inflammatory cells. H.–E., ×140

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COLONIZATION OF INFANT MICE BY K99+ *ESCHERICHIA COLI* AND LACK OF COLONIZATION BY *STREPTOCOCCUS FAECIUM* M74

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Intragastrically inoculated infant mice were colonized by enterotoxigenic *Escherichia coli* (ETEC) 0101 : K30, K99 : NM strains but not by nonenterotoxigenic *E. coli* 08 : K50 : NM strain. Adhesion of the K99+ ETEC strain to the small intestinal epithelium of mice was demonstrated by immunofluorescence and by transmission electron microscopy.

Streptococcus faecium M74 did not colonize the small intestine of infant mice and did not prevent colonization by K99+ ETEC.

Keywords. Colonization, infant mice, *Escherichia coli*, K99+, *Streptococcus faecium* M74.

Colonization of the bovine and porcine small intestine by enterotoxigenic *Escherichia coli* (ETEC) possessing the adhesive antigen K99 is widely recognized (Moon et al., 1977; Moon et al., 1976; Moon et al., 1980; Ørskov et al., 1975). It has also been reported that K99+ ETEC could colonize the small intestine of newborn mice (Kétyi et al., 1978; Moon et al., 1979*a*). However, morphological evidence of the K99-mediated colonization is still lacking in infant mice.

One way to prevent this colonization is the immunization of the dam by vaccines containing K99 to provide specific colostral immunity for the newborn (Acres et al., 1979; Nagy, 1980). Other ways would be to block adhesin receptors in the small intestine or inhibit the growth of ETEC by competitive intestinal bacteria (Davidson and Hirsh, 1975; Underdahl et al., 1982; Wadström, 1984).

This paper describes experiments aimed to investigate the morphology of the small intestinal colonization of infant mice by K99+ ETEC and to prevent this colonization by a possibly competitive bacterial strain *Streptococcus faecium* M74 (Potsubay, 1983; Wadström, 1984).

Materials and methods

Animals. CF-1 (Carworth Farms) and CFLP (LATI Co., Hungary) mice of either sex, 2 to 4 days old, were used for the experiments. The infant mice were separated from their mother after being inoculated, and were kept at 37 °C unless otherwise stated.

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Bacterial strains. *E. coli* 431 and 613 (both: 0101 : K30 : NM : K99$^+$, ST$^+$) were used as K99$^+$ ETEC strains, *E. coli* 124 (08 : K50 : NM) was used as nonenteropathogenic *E. coli* (NEEC). *Streptococcus faecium* M74 (NCIB 1181, kindly provided by Monopharm Co., Hungary) was used in trypticase soy broth (TSB) culture or as a rehydrated milk grown product (Monospray) for competitive colonization trials "A" and "B", respectively.

Mode of infection. All strains of bacteria were inoculated intragastrically via a stomach tube. Bacterial suspensions were grown at 37 °C in TSB, unless otherwise stated. The dried, milk grown streptococcus culture was rehydrated adding 5 g dry material to 40 ml PBS.

Competitive colonization experiments. Mice were inoculated (pretreated) with log 8.11 bacteria of *Streptococcus faecium* M74 grown in TSB (experiment "A") or with log 7.38 *Streptococcus faecium* M74 in rehydrated milk grown product (experiment "B"). Controls were left without pretreatment. Both pretreated and control mice were kept at 37 °C for 12 h, and inoculated with K99$^+$ ETEC 613 (log 6.43 and 6.15 bacteria, respectively). Mice of both groups in both experiments were kept further for another 12 h at 37 °C before euthanasia and removal of the small intestine.

Bacterial counts of the inocula and small intestine were tested by standard methods. Entire murine small intestine was placed and vortexed in 50 ml cold PBS (Universal Laboratory Aid, Type 309) at approximately 10,000 rpm for 5 min and colony-forming units (CFU) were counted in 0.1 ml of appropriate tenfold dilutions, on blood agar for *E. coli* and on Edwards' blood agar for *S. faecium*.

Statistical analysis. A method of "*t*" statistics for two means was used to analyse the data.

Morphology. Immunofluorescent (association index) studies were performed on cryostate sections of the small intestine using FITC-conjugated anti 0101 : K30 : K99 and anti 08 : K50 serum, as described earlier (Nagy et al., 1976). Transmission electron microscopy of ultrathin section of the small intestine was also done by earlier methods (Nagy et al., 1976). For these studies log 9.0 *E. coli*/mouse of K99$^+$ ETEC 431 and NEEC 124 were inoculated and mice were kept at approximately 25 °C for 24 h before sampling.

Results

Six mice inoculated with K99$^+$ ETEC 431 showed high (mean 4) association index; large numbers of fluoresceinating bacteria were detected on the small intestinal villi and very few in the lumen (Fig. 1), while no bacteria could be detected in the small intestine of another six mice inoculated with NEEC 124, resulting in an association index of 1. In ultrathin sections of the

small intestine of a mouse colonized by the K99+ ETEC, these bacteria were detected near the intact microvilli, with several fuzzy surface appendages (Fig. 2).

Further colonization studies were performed using K99+ ETEC 613, NEEC 124 and *S. faecium* M74 bacteria, inoculating 24, 24 and 12 mice with $\log_{10}$ 6.45, 6.74 and 6.32 bacteria, respectively, as described in the methods. Mean CFU of the small intestinal bacteria of the inoculum are presented on Fig. 3. The small intestinal count of K99+ ETEC was significantly ($P < 0.001$) greater than that of NEEC. Also the latter was significantly ($P < 0.001$) higher than that of *S. faecium* M74.

Results of the competitive colonization experiments are presented in Table I. According to these data, the small intestinal number of K99+ ETEC in both the streptococcus-treated and in the nontreated groups was practically the same (in experiment A), or very similar (in experiment B). The small intestinal count of *S. faecium* colonies was about one log below that of K99+ ETEC, in both experiments.

Discussion

Colonization of infant mice by K88+ ETEC and competitive blocking of K88 receptors in the murine small intestine was indirectly demonstrated by Davidson and Hirsh (1975). Previous findings (Kétyi et al., 1978; Moon et al., 1979*a)* also indicated that the small intestine of newborn mice can be

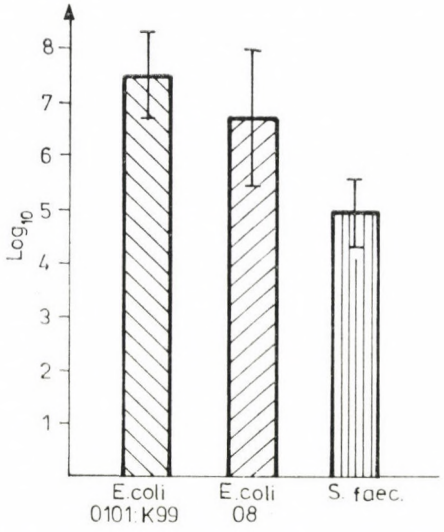

Fig. 3. Mean colony forming units (CFU) of K99+ ETEC (0101 : K30 : K99), NEEC (08 : K50) and of *S. faecium* M74 bacteria in the entire small intestine of newborn mice 12 h post-inoculation. Bar represents standard deviation

Table I

Total small intestinal colony-forming units (CFU) of K99+ ETEC and of *S. faecium* M74 in mice with and without *S. faecium* pretreatment

Experiment	Strains of the inocula	Number of mice tested	CFU of the inocula (log 10)	CFU of the small intestine (log 10)			
				K99+ ETEC		*S. faecium**	
				$\bar{x}$	Sx	$\bar{x}$	Sx
A	*S. faecium* (in TSB) + + *E. coli* 0101 : K30 : K99	10	8.11 6.43	7.39	±0.49	6.26	±0.75
	E. coli 0101 : K30 : K99	10	6.43	7.42	±0.92	6.35	±0.77
B	*S. faecium* (in milk) + + *E. coli* 0101 : K30 : K99	5	7.38 6.15	6.39	±0.08	5.04	±0.04
	E. coli 0101 : K30 : K99	5	6.15	5.76	±0.02	4.76	±0.02

* *S. faecium*-like colonies; $\bar{x}$ = mean; Sx = standard deviation.

colonized by ETEC carrying K99. The present morphological observations on the strong association of K99+ ETEC to the murine small intestinal epithelium are similar to those described for K99+ ETEC in pigs earlier (Moon et al., 1979*b*; Moon et al., 1977), and suggest that this colonization is also mediated by the antigen K99. Runnels et al. (1980) reported that K99+ ETEC adhered to the intestinal epithelial cells of infant mice in vitro. This work demonstrated that the same occurs in vivo as well. In view of the adhesive nature of colonization of mouse intestine by K99+ ETEC demonstrated in this work, K99 ETEC-infected infant mice appear to be an appropriate model to study pilus-mediated colonization and disease caused by ETEC.

S. faecium M74 was thought to compete with this K99-mediated colonization either by adhesion to the small intestinal epithelium or by some anti-*E. coli* activity as described for porcine ETEC strains by Underdahl et al. (1982) and for human ETEC strains by Wadström (1984).

The experiments reported here demonstrated that the strain *S. faecium* M74 did not intensively colonize the small intestine of newborn mice. Pretreatment with *S. faecium* did not inhibit colonization of K99+ ETEC.

S. faecium M74 is originated from the intestinal flora of a healthy child. Therefore, it could have adhesion factors that match with receptors on the small intestine of human and possibly of some animal species other than the mouse, like the strain *S. faecium* C-68 reported by Underdahl et al. (1982). It may also be that the appropriate in vivo propagation of *S. faecium* M74 requires other inoculum substrates than applied here. These and other aspects were tested using newborn pigs (Ushe and Nagy, 1985).

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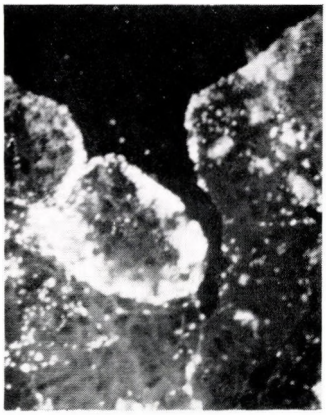

Fig. 1. Fluorescein antibody conjugated frozen section from the ileum of an infant mouse 24 h after intragastric inoculation with K99+ ETEC. Notice a heavy bacterial layer on the villi and almost no bacteria in the lumen. Approximate magnification: × 1000

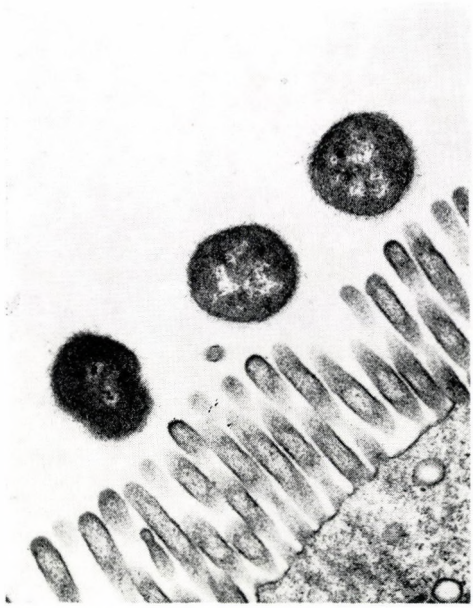

Fig. 2. Transmission electron microscopic picture from the ileum of an infant mouse 24 h after intragastric inoculation with K99+ ETEC. Bacteria with fuzzy surface appendages near the microvilli. Approximate magnification: × 27,000

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DEMONSTRATION OF CELL SURFACE ANTIGENS OF NORMAL AND VIRUS-TRANSFORMED CHICKEN LYMPHOCYTES BY THE PEROXIDASE-ANTIPEROXIDASE METHOD

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Rabbit antisera produced against chicken thymus (T), bursa (B), MDCC-HP2 cells expressing Marek's disease tumour-associated surface antigen (MATSA) and against the heavy chain of chicken IgM (μ) stained 99% of T, 91% of B, 100% of MDCC-HP2 and 7% of B or 5% of spleen cells, respectively, by the indirect immunofluorescence method on living cells. Dubious fluorescence was obtained on cells prefixed in buffered formol acetone, but well-delineated membrane-type reaction was observed on 90, 85, 93 and 2% of T, B, MDCC-HP2 and B as well as spleen cells, respectively, when the peroxidase–antiperoxidase method was used. With anti-B cell serum 67% and 77%, whereas with anti-μ heavy chain serum less than 1% and 5% of cells of a B lymphoblastoid cell line (LSCC-CU10) showed positive staining by the indirect immunofluorescence method on living cells and by the peroxidase–antiperoxidase method on fixed cells, respectively. These results indicate that characteristic cell surface markers of normal and virus-transformed chicken lymphocytes, demonstrable by the indirect immunoflourescence method on living but not on fixed cells, can be consistently detected by the peroxidase–antiperoxidase procedure even on properly fixed smeared cells.

Keywords. Chicken, cell surface antigens, detection, T lymphocytes, B lymphocytes, T and B lymphoma cells, immunofluorescence test, immunoperoxidase test, comparison.

Although immunofluorescence (IF) methods are reliable techniques for the demonstration of surface antigens of chicken lymphocytes (Albini and Wick, 1974; Cooper et al., 1974; Hudson and Payne, 1973; Ikuta et al., 1981; Nazerian and Sharma, 1975; Payne and Rennie, 1975; Powell et al., 1974; Witter et al., 1975), there has been considerable interest in applying immunoperoxidase procedures to immunohistochemistry (Sternberger, 1979; Taylor, 1978). Namely, some of the limitations of the IF methods (impermanence and poor morphology of preparations, requirement for living cell suspensions and an expensive fluorescence microscope, and incompatibility with the routine histological procedures) can be eliminated by using the unlabelled antibody enzyme method (peroxidase–antiperoxidase system, PAP) of Sternberger et al. (1970). With this procedure Mason et al. (1975, 1977) demonstrated surface and cytoplasmic immunoglobulins, as well as other cellular antigens on properly fixed human leucocytes, whereas Bross et al. (1978) could detect surface antigens only on living, but not on fixed lymphocytes. Hoffmann-Fezer and Hoffmann (1980) studied thymus (T) and bursa (B) cell distribution in cryostate-sectioned and acetone-fixed Marek's disease virus induced lesions with the PAP method.

The purpose of the present experiments was to investigate whether surface antigens of normal chicken T and B cells, as well as of lymphoblastoid cell line cells from Marek's disease (MD) and lymphoid leukosis tumours, demonstrable on living cells with the membrane IF method, can be demonstrated on prefixed smeared cells with the IF and/or with the PAP method. This report describes the results of testing rabbit antisera against characteristic surface antigens of normal and virus-transformed chicken lymphocytes simultaneously in the indirect IF and PAP procedure. The sensitivity of both techniques has been compared by staining live and prefixed cells of identical samples. The results indicate that these antigens were consistently detected on fixed cells with the PAP method, while the results obtained with the IF technique were either negative or inconclusive.

Materials and methods

Lymphoblastoid cell lines

MDCC-HP2 (Powell et al., 1974) and MDCC-RPl (Nazerian et al., 1977), two lymphoblastoid cell lines expressing MD tumour-associated surface antigen (MATSA; Witter et al., 1975), were obtained through the courtesy of P. C. Powell (Houghton Poultry Research Station, Houghton, England), and LSCC-CUlO, a B lymphoblastoid cell line (Calnek et al., 1978), from B. W. Calnek (Cornell University, Ithaca, U. S. A.). The B and the MD lymphoblastoid cell lines were grown in Hahn's medium (Hahn et al., 1977) and in RPMI 1640 supplemented with 10% newborn calf serum and 10% tryptose phosphate broth, respectively. The cells were subcultured at 48-h intervals.

Preparation of cell suspensions

Thymus, bursa of Fabricius or spleen cell suspensions were prepared by gently teasing apart minced organs in ice-cold phosphate-buffered saline (PBS, 0.01 M, pH 7.2), containing 10% bovine serum. The resulting cell suspensions were filtered through a stainless-steel mesh and washed with ice-cold PBS three times by centrifugation. The proportion of viable cells was estimated by trypan blue exclusion, and the suspension was adjusted to a final concentration of $2–4 \times 10^7$ cells/ml.

Production of anti-T cell and anti-B cell rabbit immune sera

Anti-T cell and anti-B cell sera were produced in rabbits according to Hudson and Payne (1973). Two groups of 5 rabbits received two intravenous injections of about 10^9 living thymus or bursa cells from 3 weeks old SPF White Leghorn chickens with a two weeks' interval, and bled one week after the last inoculation. The obtained antisera were inactivated at 56 °C for

45 min and consecutively absorbed with washed and packed chicken red blood cells, glutaraldehyde-insolubilized chicken liver homogenate, chicken serum globulin and bovine serum. The anti-T cell serum was further absorbed with B cells and the anti-B cell serum with T cells from 4–6 weeks old SPF White Leghorn chickens.

Production of anti-MATSA rabbit immune serum

This antiserum was produced in rabbits against MDCC-HP2 cells according to Powell et al. (1974). The antiserum was absorbed with chicken red blood cells, chicken liver homogenate, chicken serum globulin and bovine serum, as well as with thymus, bursa and spleen cells of 6–8 weeks old SPF White Leghorn chickens until it became completely nonreactive with normal chicken lymphocytes.

Production of anti-chicken IgM rabbit immune serum

Rabbits were injected intradermally with chicken IgM in a dose of 25 μg/kg body mass emulsified in 200 μl incomplete Freund's adjuvant five times fortnightly. Six weeks after the last inoculation, rabbits were boosted with the same antigen dose and bled 10–14 days later.

Linking antiserum

Anti-rabbit IgG sheep immune serum, used as linking antibody for the PAP method, was prepared as described previously (Németh, 1972).

Preparation of peroxidase-antiperoxidase complex

Soluble complex of peroxidase and rabbit anti-peroxidase antibody was prepared and characterized as described by Sternberger (1979), using Sigma type VI horse-radish peroxidase with RZ values between 2.8 and 3.1 (Sigma Chemical Co., St. Louis, Missouri, U. S. A.). It contained 3.3 mg/ml of protein, and had a peroxidase : antiperoxidase mole ratio of approximately 2 : 1.

Purification of chicken immunoglobulins

IgG was purified from egg yolk. Egg yolk globulin, separated as described (Jensenius et al., 1981), was dialysed against 0.05 M Tris-HCl + 0.06 M NaCl, pH 7.5, buffer and applied to a DEAE-Sephacel (Pharmacia Fine Chemicals AB, Uppsala, Sweden) column equilibrated with the same buffer. The IgG fraction that passed straight through the column was further purified by agarose gel electrophoresis (Johansson, 1972).

IgM, as prepared from adult chicken serum (Benedict, 1967; Leslie and Benedict, 1968), was found to be contaminated with IgG, IgA and several other serum proteins. Therefore the final purification was made on an im-

munoadsorbent column prepared by coupling chicken IgM class-specific rabbit antibodies to CNBr-activated Sepharose 4B.

IgA was isolated from bile (Watanabe and Kobayashi, 1974) and further purified by immunoaffinity chromatography using a chicken IgA class-specific rabbit IgG antibody-coupled CNBr-activated Sepharose 4B column.

The immunoglobulin preparations were found to be pure when tested at 5–10 mg/ml concentration by agarose gel electrophoresis (Johansson, 1972) and two-dimensional immunoelectrophoresis (Crowle, 1977) against a polyvalent rabbit antiserum to chicken serum.

Immunoadsorbents

For the removal of unwanted antibodies from rabbit antisera, immunoadsorbents were prepared from bovine serum, chicken liver homogenate, chicken serum globulin, egg yolk globulin and chicken bile IgA by polymerization of proteins with glutaraldehyde. Immunoadsorption was performed according to the batch procedure (Ternynck and Avrameas, 1976).

Indirect haemagglutination

Passive haemagglutination was performed on microtitration plates (Takátsy, 1955). Glutaraldehyde-fixed tanned sheep red blood cells were prepared and coated with chicken IgG, IgM or IgA as described by Bing et al. (1967).

Preparation of cell smears

Cell suspensions from thymus, bursa and spleen of 4–6 weeks old SPF White Leghorn chickens and from lymphoblastoid cell lines were washed three times by centrifugation in ice-cold PBS, containing 0.02% EDTA and 0.2% bovine serum albumin. Six drops of about 5 mm in diameter of the cell suspensions were placed onto a clean microscope slide by means of a 200-μl Gilson pipette. Excess fluid was withdrawn to leave only a very thin film of cell suspension. The smears were air-dried under a fan, fixed in buffered formol acetone for 30 sec at room temperature (Mason et al., 1975), or in ice-cold acetone for 15 min, rinsed in distilled water, and either processed immediately according to the IF or PAP method, or air-dried and stored frozen on silica gel at — 70 °C for later use.

Immunofluorescence staining

Membrane indirect IF staining of intact living cells was performed according to Hudson and Payne (1973). Staining of fixed cell smears was carried out as described (Goldman, 1968). Fluorescein-isothiocyanate-conjugated swine anti-rabbit IgG (SEVAC, Prague, Czechoslovakia) was used at 1 : 20 dilution. All observations were made with incident light illumination on a Fluoval-2 microscope (Carl Zeiss, Jena, GDR).

PAP staining procedure

Staining according to the PAP system was carried out as described by Boenisch (1980). As diluent, 0.05 M Tris-HCl + 0.15 M NaCl, pH 7.6 buffer, containing 1% normal sheep serum was used, and the plain buffer served for washing. Prefixed cell smears were stained with primary antiserum, sheep anti-rabbit IgG linking antiserum and rabbit PAP, and the peroxidase reaction developed with DAB susbstrate, consisting of 0.05% 3,3'-diaminobenzidine tetrahydrochloride and 0.01% H_2O_2 in 0.05 M Tris-HCl, pH 7.6, buffer. Normal sheep serum was used to block non-specific binding before administration of the primary antiserum. The slides were either counterstained by the May-Grünwald–Giemsa method or mounted in buffered glycerol (7 : 3 mixture of glycerol and glycine buffer, pH 8.6). The PAP complex and the linking antibody were previously titrated on a broad range of primary antiserum dilutions, and they were finally set at 1 : 20 working dilution. To check specificity, staining of each type of target cells was performed by sequentially replacing primary antisera, linking antibody and PAP complex with diluting buffer during the procedure. Staining with DAB substrate alone served as additional control. Because there was no staining in any case with DAB substrate alone after blocking endogenous peroxidase activity and no evidence of non-specific binding of either linking antibody or PAP complex to any of the target cells used, normal rabbit serum at a tenfold dilution was routinely used as control throughout the further experiments.

Experimental design

Anti-T cell, anti-B cell, anti-MATSA and anti-μ heavy chain rabbit immune sera were chessboard-titrated on T, B, spleen and LSCC-CUlO cells, as well as on MATSA-positive MDCC-HP2 and MDCC-RP1 cells with the IF method on live cell suspensions. From the same cell suspensions several fixed cell smears were prepared and stained according to the IF and/or to the PAP method. Proportions of positive cells were estimated by counting 150–200 leucocytes per preparation.

Results

Characterization of anti-T cell serum

Results of a representative titration are shown in Fig. 1. After four absorptions with B cells, anti-T cell serum diluted 1 : 20 stained about 100, 78 and 16% of T, spleen and B cells, respectively, while the 1 : 40 dilution of the same serum gave ring- and/or crescent-shaped membrane fluorescence on 84, 82 and nil % of T, spleen and B cells, respectively, by IF staining on live cell suspensions. Thymus-dependent antigen of MDCC-HP2 cells could also be demonstrated on about 72% of cells with 1 : 10 serum dilution, but in this case 35% of B cells were also positive. When smeared cells were fixed in buf-

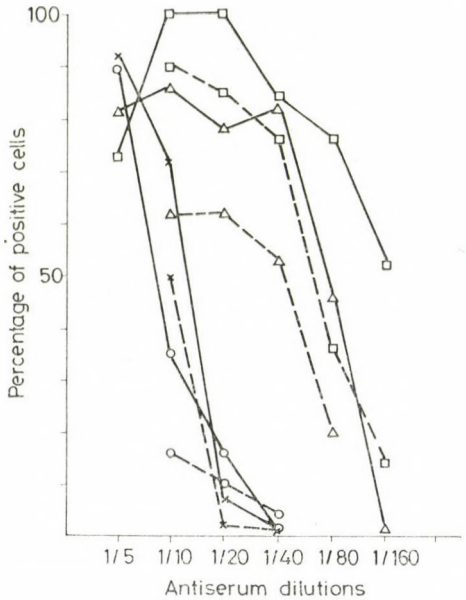

Fig. 1. Titration of anti-T cell rabbit immune serum by the IF and PAP methods. Percentage of positive thymus (□), bursa (○), spleen (△) and MDCC-HP2 (*) cells stained by the IF method on live cell suspensions (continuous line) and by the PAP method on formol acetone-fixed cell smears (broken line)

fered formol acetone or in ice-cold acetone, the result of IF staining was either negative or equivocal, even if the highest antiserum concentration (1 : 5 dilution) was used.

Prefixed smeared cells were consistently stained by the PAP method. The intensity of specific staining was optimal with buffered formol acetone fixation. At 1 : 10 antiserum dilution, 90, 16, 50 and 62% of T, B, MDCC-HP2 and spleen cells, respectively, showed staining (Fig. 1). The reaction product developed mainly at the cell periphery as a smooth dark-brown ring leaving the cell center over the nucleus more or less transparent. Such a ring pattern of staining is considered as typical membrane-type reaction (Fig. 2). After fixation in plain acetone, positive PAP staining was observed only on T cells. The reaction product deposited over the cell surface was hairy or villous and also the cytoplasm showed diffuse intense brownish staining (Fig. 3). There was no staining at all on cells smeared in plain PBS and fixed with either fixative.

Monospecific anti-T cell serum gave a typical dilution plateau by both the IF and PAP method, although the latter was somewhat lower (Fig. 1).

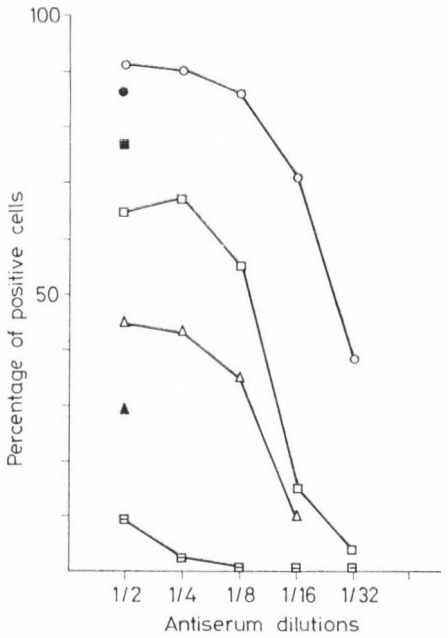

Fig. 4. Titration of anti-B cell rabbit immune serum by the IF and PAP methods. Percentage of IF-positive bursa (○), thymus (⊟), spleen (△) and LSCC-CU10 (□) cells on live cell suspensions and that of PAP-positive bursa (●), spleen (▲) and LSCC-CU10 (■) cells on formol acetone-fixed cell smears

Characterization of anti-B cell serum

Results of titration experiments on B, T, spleen and LSCC-CU1O cells are given in Fig. 4. This antiserum at a dilution of 1 : 4 gave characteristic granular membrane fluorescence on about 90, 43, 67, 2.5 and nil % of B, spleen, LSCC-CU1O, T and MDCC-HP2 cells, respectively, with IF staining on living cells. Fixed cells were negative, as it was observed with anti-T cell serum.

When buffered formol acetone-fixed cells were subjected to PAP staining, extensive cross-reaction with T cells was observed. On further absorption with T cells, insolubilized bovine serum and chicken serum globulin immunoadsorbents, T cell reactivity disappeared. The activity of the antiserum became, however, even weaker, and it could be used only at a twofold dilution. It stained 85% of B, 28% of spleen and 77% of LSCC-CU1O cells. Reactive B cells showed a dark-brown ring linear or patchy pattern of surface staining (Fig. 5). No staining was observed after fixation of smears with ice-cold acetone, or when cells were smeared in plain PBS.

Dilution plateau was observed by titration of antiserum with the IF method. It should be noted, however, that either the IF or PAP method was used, results of staining with anti-T cell serum and anti-B cell serum were

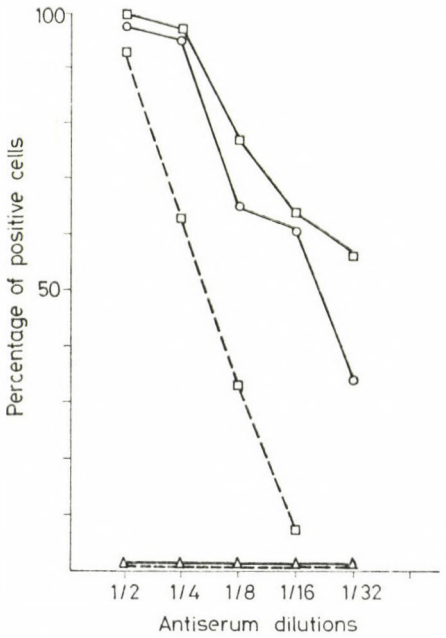

Fig. 6. Titration of anti-MATSA rabbit immune serum by the IF and PAP methods. Percentage of positive MDCC-HP2 (□), MDCC-RP1 (○) and thymus, bursa, spleen, LSCC-CU10 (△) cells stained by the IF method on live cell suspensions (continuous line) and by the PAP method on formol acetone-fixed cell smears (broken line)

clear-cut on central T and B cells, respectively, but with peripheral T and B cells the number of positive cells and the intensity of staining progressively decreased with increasing cell numbers (higher than 2×10^6) used for staining.

Characterization of anti-MATSA serum

Fig. 6 shows the results of titration with T, B, spleen, LSCC-CU1O cells and MATSA-positive MDCC-HP2 and MDCC-RP1 cells. This antiserum stained about 100% of living MDCC-HP2 and MDCC-RP1 cells by the IF method, but there was no staining on T, B, spleen and LSCC-CU10 cells. No reaction was seen on MATSA-positive smeared cells after fixation with either fixative.

When buffered formol acetone-fixed cell smears were stained by the PAP method, about 90% of MDCC-HP2 cells showed ring-shaped dark-brown granular deposition of the reaction product over the cell surface, a typical membrane-type reaction (Fig. 7). No staining was found on cells smeared in plain PBS or on acetone-fixed cell smears.

Characterization of anti-chicken μ heavy chain antiserum

Anti-chicken IgM immune serum, obtained by immunization of rabbits with pure IgM, heavily cross-reacted with IgG and IgA (Fig. 8A). To render

the antiserum IgM class-specific, it was repeatedly absorbed with glutaraldehyde-polymerized egg yolk globulin and bile IgA immunoadsorbents. The specificity was initially checked by immunodiffusion against a wide concentration range of chicken IgG and IgA. The final check was made by two-dimensional immunoelectrophoresis and passive haemagglutination. The antiserum was considered IgM class-specific (μ heavy chain specific) when it developed a single precipitation loop with adult chicken serum upon two-dimensional immunoelectrophoresis (Fig. 8B) and became completely nonhaemagglutinating against sheep red blood cells coated with the heterologous class of immunoglobulins.

This antiserum was further tested by both IF and PAP techniques on live and fixed cells used previously for the characterization of the other antisera. Cell surface IgM was demonstrated on about 7% of B, 5% of spleen, but on less than 1% of LSCC-CU10 cells by the IF method on living cells, using an antiserum dilution of 1 : 40. The result with buffered formol acetone-fixed cells was equivocal, but the percentage of positive cells showing strong cytoplasmic fluorescence after acetone fixation was very close to that obtained with intact living cells.

On PAP staining of buffered formol acetone-fixed cell smears, about 2% of B and spleen cells showed a more or less complete dark-brown ring around the cell periphery (Fig. 9), while in the case of about 5% reactive LSCC-CU10 cells, the dark-brown reaction product covered almost the whole cell (Fig. 10). After acetone fixation, the typical membrane-type staining pattern of B and spleen cells switched to a typical cytoplasmic-type reaction, i.e. the reaction product covered the cytoplasmic but not the nuclear area (Fig. 11).

Controls

Control preparations, in which normal rabbit serum or unrelated antisera were substituted for specific primary antisera, were competely negative by both staining techniques irrespective of the serum–target cell combination. Only among acetone-fixed spleen and bursal cells were there a very few cells (about 0.4–0.6% of the cells in some preparations) showing a diffuse and pale cytoplasmic reaction after PAP staining.

Discussion

Using the indirect membrane IF method, our anti-T cell and anti-B cell antisera stained conclusively high percentage of live thymic and bursal cells, respectively. However, when spleen cells were used as target, the proportion of reactive cells and the intensity of staining depended on the number of cells used, presumably because of the low specific antibody content of the

antisera. A possible reason for this may be that rabbits used for immunization responded mainly against surface antigens of central T or B cells and poorly against those present on peripheral or on both central and peripheral lymphocytes. Alternatively, some of the specific antibodies reacting against common surface antigens of central and peripheral T or B cells (Schauenstein, 1979) were removed during the absorption procedure, as erythrocyte suspensions used for antiserum absorption were never depleted of peripheral leucocytes. Similar results were obtained by Hudson and Payne (1973) who reported that some of their anti-T cell sera reacted exclusively with central thymus cells. Nevertheless, our antisera can be used for the identification of T and B lymphocytes.

Our anti-MATSA antiserum reacted with a high percentage of MATSA-positive cells (Powell et al., 1974; Witter et al., 1975) by both the IF method on live cells and the PAP method on fixed smeared cells.

The anti-μ heavy chain antiserum, scrupulously tested for specificity by a variety of immunochemical methods, readily detects membrane-bound and cytoplasmic IgM by the IF and the PAP method. In contrast to a previous report (Schat et al., 1982), 5% of LSCC-CU10 cells were IgM-positive. This cell line was established (Calnek et al., 1978) from Olson's transplantable lymphoid leukosis tumour (Olson, 1941), and therefore most of the cells should be IgM-positive unless they have lost their IgM-producing capacity during the in vitro passages. We observed a relatively low percentage of IgM-positive cells among normal bursa and spleen cells (Neumann and Witter, 1979), presumably because of the real class-specificity of our antiserum. Truly, the declaration of monospecificity of an anti-immunoglobulin antiserum depends on the sensitivity of the test system (Hijmans et al., 1969; Preud'Homme and Labaume, 1975).

It is worth noting that we failed to isolate immunochemically pure IgM by the commonly used physicochemical methods (Benedict, 1967; Leslie and Benedict, 1968). As it is well known (Lebacq, 1979), the rabbit antiserum produced against pure IgM strongly cross-reacts with the other classes of chicken immunoglobulins, which could be completely eliminated by repeated absorption with insolubilized egg yolk IgG and bile IgA immunoadsorbents.

Immunoperoxidase techniques have seldom been used for the visualization of cell surface antigens on chicken lymphocytes (Hoffmann-Fezer and Hoffmann, 1980). It is clear from the results of the present experiments that the PAP technique is a reliable and reproducible method in detecting immunoglobulin and other antigenic markers on normal and neoplastic chicken lymphoid cells. There was no background colouring, and control preparations, except a rather few bursal and spleen cells, were consistently negative whatever the sample under study. Besides, the results of chessboard titrations of specific primary antisera against both homologous and heterologous cells proved un-

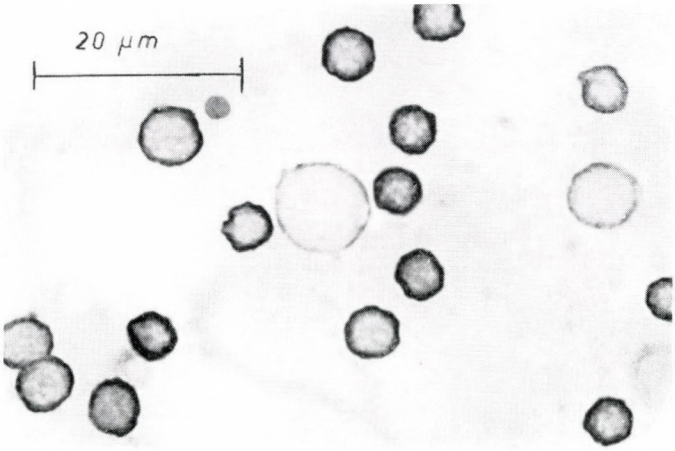

Fig. 2. Buffered formol acetone-fixed thymus cells stained with anti-T cell serum by the PAP method. Positive cells stained in rings; no nuclear counterstaining

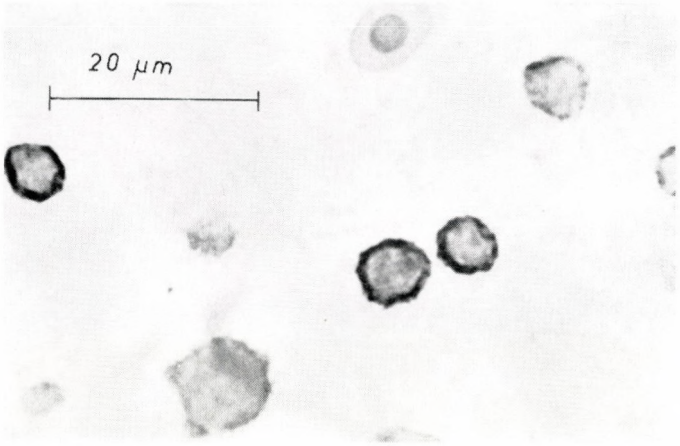

Fig. 3. Acetone-fixed thymus cells stained with anti-T cell serum by the PAP method. Positive cells show villous surface and intense cytoplasmic staining

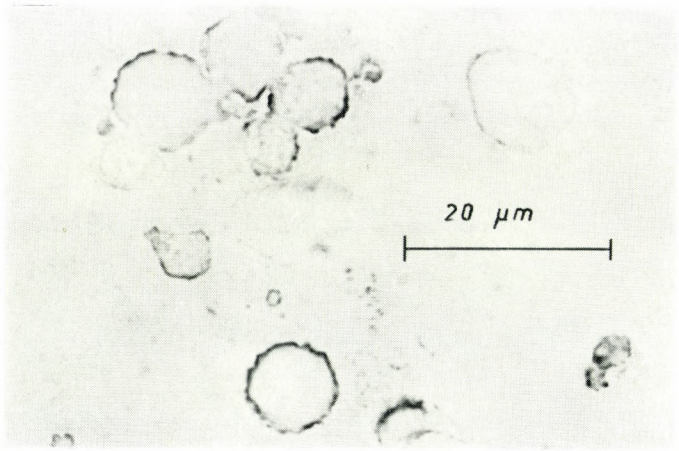

Fig. 5. Bursal cells labelled with anti-B cell serum by the PAP method on buffered formol acetone-fixed cell smear. Positive cells show patchy pattern of surface staining; no nuclear counterstaining

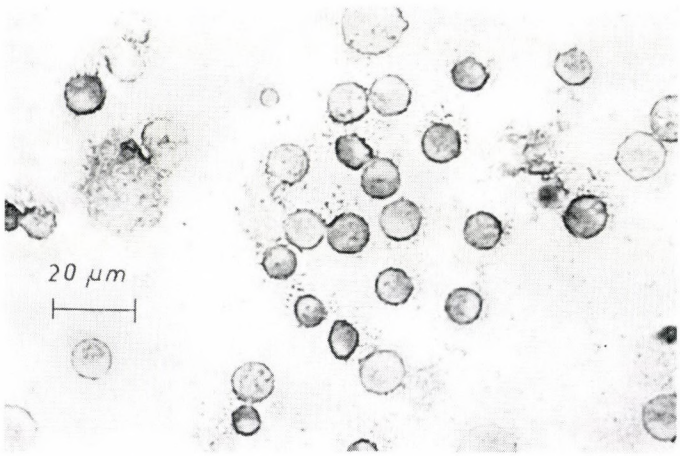

Fig. 7. MDCC-HP2 cells stained for MATSA by the PAP method on buffered formol acetone-fixed cell smear. Positive cells show more or less complete ring-staining of the cell periphery

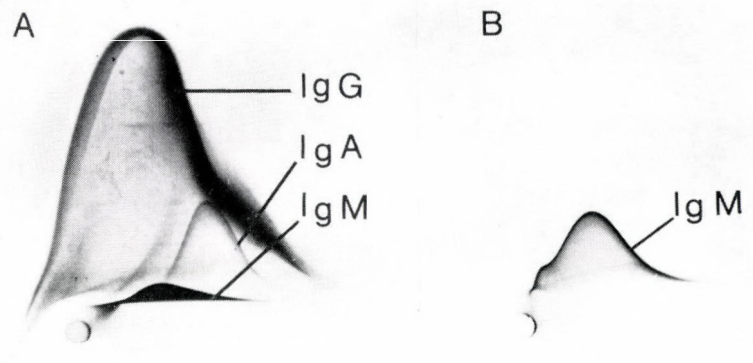

Fig. 8. Two-dimensional immunoelectrophoresis of anti-chicken IgM (A) and anti-chicken μ heavy chain (B) rabbit antiserum against normal chicken serum. Antiserum concentration: 10 μl per cm^2 of gel. In the first dimension 2 μl of pooled normal chicken serum was separated at 7 V cm^{-1} for 50 min. Anod to the right. Second dimension electrophoresis was performed at 2 V cm^{-1} for 20 h. Anod at the top

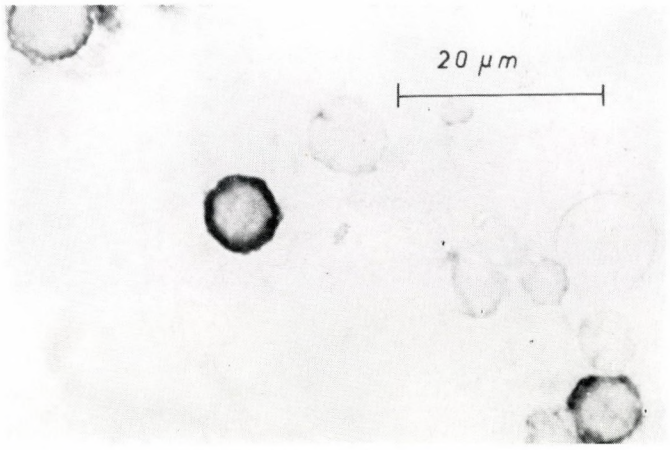

Fig. 9. PAP staining of buffered formol acetone-fixed spleen cells for IgM. Note positive cell showing intense ring-staining of the cell surface; no nuclear counterstaining

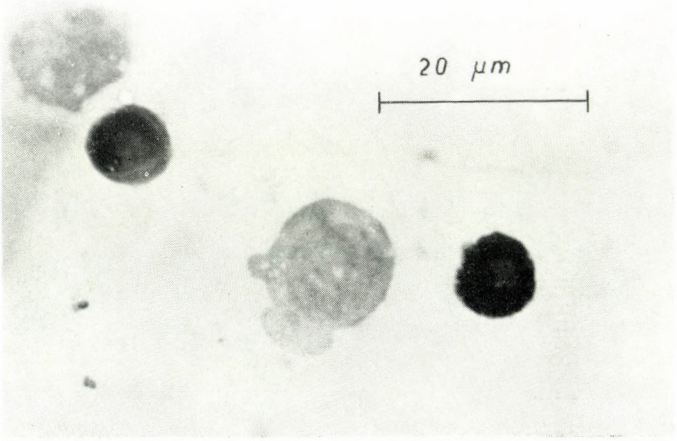

Fig. 10. PAP staining of buffered formol acetone-fixed LSCC-CU10 cells for IgM. Note the two positive cells stained diffusely

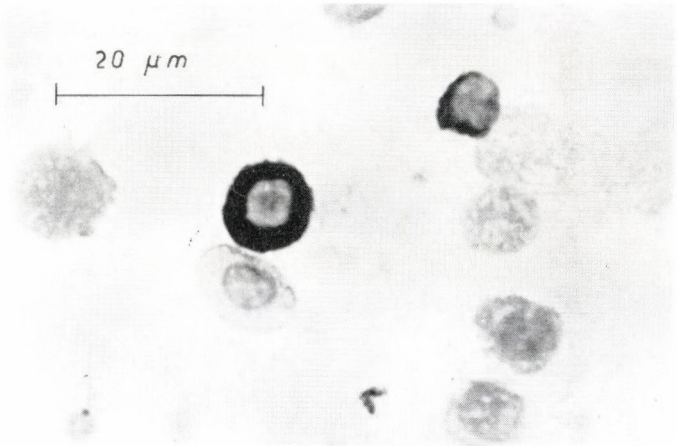

Fig. 11. PAP staining of acetone-fixed spleen cells for IgM. Note a cell showing strong diffuse cytoplasmic staining

doubtedly the specificity of the staining. The reasons for the faint diffuse cytoplasmic staining of some bursal and spleen cells in control preparations reacted with normal rabbit serum instead of specific primary antisera were not fully explored. It seems probable, however, that this type of staining arose from nonimmunologic binding of some rabbit immunoglobulins to certain unknown chicken cellular components, though the presence of antibodies of unknown specificities within the serum used, including naturally occurring Forssman-type antibodies reacting against chicken tissue constituents, cannot be excluded.

We have not conducted detailed studies on the suitability of a large panel of different fixatives for PAP staining. Of the two fixation methods employed, brief fixation of cell smears with buffered formol acetone according to Mason et al. (1975) provided better results. The specific PAP staining pattern of each type of buffered formol acetone-fixed target cells was a distinctive membrane-labelling: the visible cell surface was clearly delineated by a dark-brown margin contrasting with a pale cytoplasm. The single exception was represented by LSCC-CU10 cells stained for IgM. These B lymphoblastoid cells stained diffusely, i. e. the peroxidase reaction product covered the whole cell including the nuclear area, unlike other IgM-positive bursal and spleen cells, where cell surface or cytoplasmic localization of IgM was easily differentiated depending on the choice of fixative. Such pattern of staining has been previously observed on human hairy leukaemia and Burkitt's lymphoma cells and said to be a type of cell surface immunoglobulin staining (Laurent et al., 1982). Following acetone fixation, specific cell surface determinants were detectable exclusively on central T lymphocytes. The staining pattern differed, however, from the characteristic staining pattern seen after buffered formol acetone fixation. In contrast, cytoplasmic IgM was readily detected, indicating that cold acetone is not a proper fixative if antigenic surface markers are to be visualized, but it is suitable for the fixation of cytoplasmic IgM. It should be noted that the specific PAP staining varied in intensity from cell to cell according to the number of antigens on the cells and the antibody titre of the antiserum used, though recognition of a positive reaction was always possible.

The proportion of PAP-positive lymphocytes tended to be somewhat lower than that revealed by IF staining of viable cells, though the PAP technique has been reported to be 100 to 1000 times more sensitive (Sternberger, 1979). Studying the presence of immunoglobulins on peripheral human leucocytes, Mason et al. (1977) also observed that the PAP technique detects about 50% less IgM-positive cells than the IF method. They suggested, with no experimental proof, however, that the technique detected only a sub-set of IgM-bearing B cells. The interpretation of their results is complicated by the fact that surface Fc receptors present on human leucocytes may bind animal immunoglobu-

lin molecules (for review, see Taylor, 1978; Sternberger, 1979). However, the Fc-mediated binding of immunoglobulins cannot be the source of discrepancy in our system, as chicken Fc receptors do not bind rabbit IgG (Nowak et al., 1978; Morton et al., 1980). It has been demonstrated that prefixation of cells is likely to reduce the sensitivity of any method of immuno-staining as a result of fixation-induced alteration of antigens (Taylor, 1978; Sternberger, 1979; Laurent et al., 1982). It is highly probable, therefore, that some or most of the surface-exposed antigens on a certain proportion of cells were destroyed, and the few intactly survived ones were not sufficient in number to be demonstrable with our antisera. Well-controlled additional experiments are needed, however, to clarify whether the difference in the proportion of reactive cells in the two immuno-staining procedures is due to the adverse effects of the used fixative on antigens or to other factors.

In conclusion, although the PAP method is relatively time-consuming and tedious to perform, its high sensitivity makes it a very useful tool for the visualization of membrane-bound and cytoplasmic IgM, as well as of other antigenic surface markers of normal and virus-transformed chicken lymphocytes on properly fixed cell smears.

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REACTION OF THE THYMUS AND THE BURSA OF FABRICIUS IN CHICKENS INOCULATED WITH *CORYNEBACTERIUM PARVUM*

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Rock-Cornish chickens aged 5 days were inoculated subcutaneously with 0.1 ml *Corynebacterium parvum* suspension (0.25 mg dry matter of heat-killed bacterial cells) in a single dose. Modifications were followed at 1, 3, 8, and 15 days after administration in the bursa of Fabricius and the thymus (total protein, RNA, DNA and glycogen contents as well as organ weight), the adrenals (ascorbic acid and glycogen contents) and the blood (serum gamma globulin and leucocyte counts).

Our results show a stimulation of both lymphoid organs, occurring earlier in the bursa of Fabricius than in the thymus. An increase of serum gamma globulin content also occurs, parallelled by an increase in the number of leucocytes, indicating immunostimulation. Long-lasting modifications in the adrenals were not observed.

Keywords. *Corynebacterium parvum*, chicken, experimental infection, thymus, bursa of Fabricius, immunostimulation.

Corynebacterium parvum is a nonspecific immunostimulating agent (Halpern et al., 1963; Halpern, 1975; Woodruff, 1975) having antitoxic and antibacterial actions (Gelencsér et al., 1980; Padányi et al., 1980) and a stimulating effect on macrophages (Fauve, 1975; Réthy et al., 1978; Todoruţiu et al., 1981).

Starting from these data, in this investigation we tried to get evidence on the effects of *Corynebacterium parvum* administration on the central lymphatic organs of the chicken.

Materials and methods

Experiments were performed on Rock-Cornish (Robro 69) chickens, aged 5 days at the beginning of the experiments. The birds were kept under appropriate zoohygienical conditions and fed a concentrated fodder suited to their age. Access to food and water was ad libitum.

The chickens were divided into two groups: control (C) and treated (T). Each chicken in Group T was inoculated subcutaneously with a single dose of 0.1 ml suspension of *C. parvum* (0.25 mg dry matter of heat-killed bacterial cells), a product of the "Cantacuzino" Institute, Bucharest. Group C chickens were injected with the same volume of saline, containing 0.2‰ formaldehyde and 1 : 10,000 sodium merthiolate. The birds survived this treatment in good health.

Eight chickens of each group were decapitated at 1, 3, 8, and 15 days after injection, respectively. The blood, the bursa of Fabricius, the thymus and the adrenals were immediately processed. From the blood, leucocyte counts and the concentrations of serum gamma globulin were determined (Gornall et al., 1949; Wolfson et al., 1948). The bursa of Fabricius and the thymus were weighed, and total protein (Gornall et al., 1949), RNA and DNA (Spirin, 1958) and glycogen (Montgomery, 1957) were determined in them. The ascorbic acid content of the left adrenal (Klimov, 1957) and the glycogen content of the right one (Montgomery, 1957) were also measured.

Statistical processing included comparison of the mean values based on Student's *t* test, and calculation of percentage differences against C values (D%). The means were previously checked for homogeneity, using Chauvenet's criterium; aberrant values were eliminated.

Results

All values are calculated for fresh tissue weight.

Bursa of Fabricius (Table I).

Table I

Total protein (TP), RNA, DNA and glycogen (G) concentrations in the bursa of Fabricius and the weight of this organ (BW) in chickens inoculated with *Corynebacterium parvum* (C = control; T = treated birds; $\bar{x}$ = mean value; ± SE = standard error; D % = percentage differences against group C; P = statistical significance)

Days			1	3	8	15
TP (mg/100 g)	$\bar{x}$ ± SE	C	221.9±11.8	229.2±8.2	212.9±4.9	230.7±23.8
		T	200.2±5.6	288.1±18.8	220.8±5.3	209.3±11.7
	D %		−10	±25	+4	−10
	P		—	<0.01	—	—
RNA (mg/g)	$\bar{x}$ ± SE	C	5.3±0.3	4.4±0.1	4.0±0.4	5.4±0.5
		T	3.9±0.3	4.3±0.6	3.9±0.3	4.6±0.3
	D%		−26	−3	−2	−16
	P		<0.01	—	—	—
DNA (mg/g)	$\bar{x}$ ± SE	C	4.1±0.4	5.0±0.4	6.0±0.6	9.8±1.0
		T	5.8±0.4	5.3±0.4	10.5±0.9	8.9±0.5
	D %		+40	+5	+74	−10
	P		<0.02	—	<0.001	—
G (μg/mg)	$\bar{x}$ ± SE	C	0.88±0.1	1.1±0.5	1.2±0.4	1.1±0.4
		T	0.81±0.07	0.8±0.2	0.8±0.1	1.0±0.5
	D %		−8	−24	−31	−8
	P		—	—	—	—
BW (mg)	x ± SE	C	71.3±2.3	176.1±20.8	311.1±35.7	520.6±71.0
		T	100.0±7.0	113.8±15.6	254.0±11.5	449.3±31.5
	D %		+40	−36	−19	−14
	P		<0.001	<0.05	—	—

Total protein concentration showed a 25% increase by day 3 ($P < 0.01$). RNA showed a 26% decrease ($P < 0.01$) one day after the injection; later it returned to the control level. DNA concentration increased by 40% ($P < 0.02$) and 74% ($P < 0.001$) as tested on day 1 and 8, respectively. The glycogen concentration ran practically together in the two groups.

The weight of the organ showed an increase one day after bacterium administration (+ 40%; $P < 0.001$), a relative decrease by day 3 (−36%; $P < 0.05$), and no significant difference at other intervals.

Thymus (Table II)

A 33% increase ($P < 0.02$) of total protein as compared to the C values was seen 3 days after inoculation. RNA concentration increased by 29% ($P < 0.05$) and 67% ($P < 0.02$) by day 3 and 8, respectively. Glycogen concentration underwent no modification at any of the intervals studied.

Thymus weight showed an increase (+ 72%; $P < 0.01$) one day after the administration of *C. parvum*, a relative decrease by day 3 (− 24%; $P < 0.05$), and no significant difference was observed at the other intervals studied.

Table II

Total protein (TP), RNA, DNA and glycogen (G) concentrations in the thymus, and the weight of the thymus (TW) in chickens inoculated with *Corynebacterium parvum* (for other explanations see Table I)

Days			1	3	8	15
TP	$\bar{x} \pm SE$	C	262.2±7.3	274.5±13.5	272.0±7.0	335.5±69.4
(mg/100 g)		T	240.4±11.1	367.7±32.9	238.2±7.3	261.0±13.9
	D %		−9	+33	−13	−23
	P		—	<0.02	<0.01	—
RNA	$\bar{x} \pm SE$	C	5.3±0.4	3.5±0.3	2.1±0.2	4.7±0.7
(mg/g)		T	4.3±0.4	4.5±0.4	3.5±0.4	3.6±0.4
	D %		−18	+29	+67	−23
	P		—	<0.05	<0.02	—
DNA	$\bar{x} \pm SE$	C	8.1±0.8	9.4±1.7	6.6±1.9	9.4±2.5
(mg/g)		T	7.8±1.0	8.0±0.8	10.9±1.3	8.2±1.3
	D %		−5	−15	+65	−13
	P		—	—	<0.05	—
G	$\bar{x} \pm SE$	C	1.08±0.1	2.1±0.9	1.7±0.4	0.8±0.2
(μg/mg)		T	1.05±0.1	2.7±1.7	1.4±0.1	1.1±0.2
	D %		−4	+24	−18	+28
	P		—	—	—	—
TW	$\bar{x} \pm SE$	C	105.7±13.5	205.5±16.2	378.0±41.0	555.4±33.2
(mg)		T	182.6±23.1	158.0±13.0	378.1±29.2	521.6±95.9
	D %		+72	−24	+0	−6
	P		<0.01	<0.05	—	—

Adrenals (Table III)

No significant modification of the ascorbic acid content was seen at any of the studied intervals, as compared to the C values. A 50% increase ($P < 0.02$) observed one day after bacterium administration was the only significant change in the glycogen content.

Blood (Table III)

Significant increases of the serum gamma globulin content (+ 22%; $P < 0.01$) as well as of the leucocyte counts (+ 64%; $P < 0.001$) occurred only 15 days after inoculation.

Discussion

Two ways have been described in the literature for the immunostimulating effect of *Corynebacterium parvum*: facilitation of antibody formation by a general stimulation of the reticulo-histiocyte system (Bach, 1976), and enhancement of macrophage function (Baldwin and Byers, 1979; Falk and McGregor, 1976; Fudenberger et al., 1980; Peters, 1978; Stiffel et al., 1977; Todoruțiu et al., 1981; Todoruțiu et al., 1982). On the other hand, it is well known that the thymus and the bursa of Fabricius of the chicken possess a specific role in the humoral and cellular immunity, respectively (Bell and

Table III

Serum gamma globulin (Gg) concentrations and leucocyte counts (L) in the blood, and ascorbic acid (AA) and glycogen (G) concentrations in the adrenals in chickens inoculated with *Corynebacterium parvum* (for other explanations see Table I)

Days			1	3	8	15
Gg (mg/ml)	$\bar{x} \pm SE$	C	10.7 ± 0.5	7.7 ± 0.3	10.4 ± 0.2	6.2 ± 0.4
		T	10.6 ± 0.2	8.2 ± 0.3	10.8 ± 0.4	7.6 ± 0.3
	D %		±0	+6	+3	+22
	P		—	—	—	<0.01
L (cells/mm³)	$\bar{x} \pm SE$	C	24,512 ± 3165	28,050 ± 3365	34,428 ± 3259	22,085 ± 2518
		T	28,825 ± 3521	28,750 ± 2335	29,137 ± 3500	36,312 ± 2150
	D %		+21	+3	+13	+64
	P		—	—	—	<0.001
AA (μg/mg)	$\bar{x} \pm SE$	C	2.1 ± 0.1	3.3 ± 0.1	3.9 ± 0.1	3.1 ± 0.1
		T	1.9 ± 0.1	3.3 ± 0.4	3.3 ± 0.4	3.0 ± 0.1
	D %		—11	±0	—15	—1
	P		—	—	—	—
G (μg/mg)	$\bar{x} \pm SE$	C	2.2 ± 0.2	2.2 ± 0.4	5.9 ± 1.7	0.8 ± 0.3
		T	3.3 ± 0.4	2.7 ± 0.8	7.1 ± 1.7	1.2 ± 0.3
	D %		+50	+24	+18	+46
	P		<0.02	—	—	—

Freeman, 1971). Thus, an involvement of both the bursa of Fabricius and the thymus in the *C. parvum*-elicited immunostimulations was expected. Our results show that this is actually the case.

The reaction of the bursa of Fabricius takes place earlier than that of the thymus. This may be due to a faster ontogenetical maturation of the bursa of Fabricius, as reflected in the modifications of some parameters of both organs in control animals (Giurgea, 1977; Stefoni et al., 1971), a fact sustained also by the control data in this paper (Tables I, II and III).

The role of *C. parvum* as an adjuvant in antibody production (Gelencsér et al., 1980; Géresi et al., 1980; Padányi et al., 1980) is manifested in our experiments probably by the increase of the serum gamma globulin content. Even if this occurs only 15 days after inoculation, it may reflect an increased resistance of the organism to various infections.

The constantly observed increase of leucocyte counts may also reflect a positive effect of *C. parvum*, which activates phagocytosis (Halpern, 1975; Morland and Morland, 1977; Todoruţiu et al., 1981).

Our results show that the effect of *C. parvum* upon both lymphoid organs lasted about 8 days. This might support the usual clinical application of this bacterium in cancer, when 2 or 3 administrations per week are continued during a longer period.

Adrenal function seems not to be affected by long-lasting modifications elicited by *C. parvum* treatment.

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DEMONSTRATION IN HUNGARY OF Q FEVER ASSOCIATED WITH ABORTIONS IN CATTLE AND SHEEP

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In the present paper, demonstration in Hungary of Q fever associated with bovine and ovine abortions is reported.

In bovine abortions, the pathological picture was characterized by severe, acute, mostly haemorrhagic and necrotic placentitis, while in sheep, in addition to similar lesions of the placenta, proliferative and inflammatory changes indicative of propagation of the causative agent were also observed in certain organs of the fetus.

Coxiella burnetii was demonstrated by light microscopy in smears prepared from the placenta and, occasionally, from fetal stomach contents and the fetal vernix, it was isolated by inoculation of guinea-pigs, and its structure was studied by electron microscopy. Specific Coxiella antibodies were demonstrated in the sera of aborted dams by the complement fixation (CF) test.

Keywords. Q fever, *Coxiella burnetii*, Rickettsia, abortion, cow, sheep.

Q fever, caused by *Coxiella burnetii*, is a zoonosis known for several decades and widespread all over the world (Derrick, 1937; Romváry, 1979). Of the domestic animals, it occurs most frequently in sheep, cattle and goats; the most conspicuous clinical symptom is abortion. Humans are highly susceptible; infected domestic animals act as primary sources of infection.

Earlier, the attention was called to the occurrence of Q fever in Hungary by cases among abattoir workers. The aetiology of the disease was clarified by Farkas et al. in 1950, based upon the clinical symptoms and positive complement fixation (CF) test. Romváry (1957*a*) conducted serological surveys on a dairy farm, where, simultaneously with numerous bovine abortions, mass occurrence of disease was observed among humans as well. By the CF test, he demonstrated antibodies to *C. burnetii* in 34.1% of the stock.

The serological surveys conducted in 1979 by Romváry et al. in South-East Hungary revealed infection by the Q fever agent in several cattle and sheep populations. These authors intentionally selected farms where the incidence of abortions and stillbirths of unknown aetiology was higher than average. In cattle stocks 40% or more, whereas in sheep flocks 12 to 23% of the animals were seropositive.

We diagnosed Q fever accompanied by abortion in February 1983 for the first time by demonstrating the causative agent; since that time the condition has been diagnosed on several occasions while examining ovine and bovine fetuses and placentae in the Central Veterinary Institute.

Our observations and experiences concerning the incidence of the disease and the first Hungarian isolation of *C. burnetii* are reported in the present paper.

Materials and methods

The fetuses and placentae were subjected to gross pathological and histopathological examination.

Histopathological examination. Samples were taken from the spleen, liver, kidney, lungs, mesenteric lymph node of the fetuses, from the placenta, as well as from the above-listed organs and the brain of the inoculated guinea-pigs. The samples were fixed in 10% formalin, and frozen or embedded in paraffin. Sections were stained with haematoxylin and eosin. In addition, pathologically-altered placental parts were stained according to the PAS reaction, while liver and kidney sections with Levaditi's silver impregnation technique, to exclude abortion of leptospiral origin.

The *smears* and *impression preparations* made of the spleen, liver, lungs, kidney, stomach contents, placenta and, occasionally, the fetal vernix were stained according to Stamp and Köster, and examined in a light microscope. *Culturing of bacteria and fungi* was attempted from the same organs on common and blood agar plates, Drigalski's and Sabouraud's media, under aerobic and anaerobic conditions, at 37 °C.

Electron microscopy. Electron-microscopic examinations were performed as described earlier (Glávits et al., 1982), with samples from a sheep's placenta and the spleen and lungs of an experimentally-infected guinea-pig.

Serology. Antibodies to *C. burnetii* were demonstrated in the aborted dams' sera by the complement fixation (CF) test. The CF antigen was prepared by Dr. József Nagy, from the phase-II variant of strain Nine-Mile of *C. burnetii*. The strain was kindly provided by the Institute of Virology, Slovak Academy of Sciences. Complement fixation obtained with sera of 1 : 20, or higher, dilution was considered positive.

Isolation of C. burnetii was attempted by experimental inoculation of guinea-pigs. As inoculum, a material prepared from the organs and placenta of ovine fetuses that had proved positive for Q fever by other examinations was used, diluted 1 : 10 with saline. Each of 5 guinea-pigs was inoculated intraperitoneally with 3 ml of this material.

The guinea-pigs were killed 3 weeks after inoculation. Smears prepared from their spleens and lungs were examined for the presence of Coxiella, and the homogenate prepared from their spleens was used for inoculating further two guinea-pigs. A third passage was performed in a similar manner.

Results

In this Institute, Q fever was diagnosed for 9 ovine and 4 bovine abortions from 7 large-scale farms of 5 counties of Hungary between February 1983 and June 1984. Case histories and on-the-spot investigations indicated the occurrence of sporadic abortions in the cattle stocks; in the sheep flocks, on the other hand, large numbers of abortions occurred within a short time.

The ovine fetuses examined were aborted in the last weeks of pregnancy. In abortions of twins and triplets, occasionally one of the fetuses was mummified. In cattle, abortion took place in the last month of pregnancy.

At *gross pathological examination*, the liver of ovine fetuses was apparently swollen and hyperaemic, the lungs were darker red than normal, compact to the touch, and atelectatic. The abomasal and rumen contents were dark-green and contained large floccules. The hazelnut-sized cotyledons of placentae were dark red and covered in some places by a continuous fibrin slough, while in other areas by a fine fibrinous coating (Fig. 1). The inflammatory lesions of intercotyledonary areas were also characteristic.

In bovine abortions placental lesions predominated; a haemorrhagic or necrotic inflammation was observed (Fig. 2).

Histopathological examination of ovine placentae revealed an acute serous, fibrinous, here and there purulent, inflammation accompanied by degeneration and necrosis of the epithelial cells of cotyledons. In cases of necrotic placentitis, part of the blood vessels showed an inflammation accompanied by thrombus formation (Fig. 3). Some cotyledonary epithelial cells were swollen and their cytoplasm contained microorganisms arranged in groups or completely filling the cytoplasm and pushing the nucleus to the cell membrane. Such cells became detached in some places (Fig. 4). *Electron microscopy* of these cells revealed polymorphous, coccoid and rod-shaped organisms 0.2 to 1, sometimes 2, μm in size. Beneath the cell membrane, frequently undulatory, a plasma membrane, and within the latter a net-like, filamentous structure of varying electron-density, containing ribosomes, was found (Fig. 5).

In the liver of some ovine fetuses an incipient, or a pronounced, serous hepatitis was observed. This was accompanied by interstitial infiltration by mononuclear cells, signs of expressed erythrocytopoiesis, and activation and, in some places, focal proliferation of the RHS cells. Haemorrhages and dystrophy of circumscribed areas occurred in the liver parenchyma. In the lungs, in addition to hyperaemia and an interstitial oedema, an incipient catarrhal-purulent pneumonia, while in the trachea infiltration of the mucous membrane by mononuclear cells was seen. Reticulocyte proliferation and haemorrhages were observed in the red pulp of the spleen, and lymphoblast proliferation in the Malpighian bodies. In the lymph nodes no alteration other than hyperaemia was found.

In the examined organs of bovine fetuses lesions similar to, but mostly less pronounced than, those in ovine fetuses were found, while in the placentae signs indicative of an extensive inflammatory process accompanied by necrosis were present. Groups of Coxiella were recognizable in the vast majority of the epithelial cells of cotyledons.

By *light microscopy of the smears*, Coxiella organisms were demonstrated in ovine placentae, fetal stomach contents and, occasionally, in the fetal vernix covering the body surface. In bovine abortions, Coxiella was present in large numbers in placental smears made of the surface of cotyledons and the fibrinous exudate involving the intercotyledonary areas. The microscopic findings were characteristic and similar in both animal species: heteromorphous, fine coccoid and rod-shaped forms, sometimes short threads, present in extremely large numbers even within one visual field were seen. The organisms were Gram-negative, stained red with both Köster's and Stamp's stain, and were mostly in intracellular position, in phagocytosed state; only few of them were found dispersed in the extracellular space (Fig. 6).

In the cases mentioned above, the *bacteriological examination* of fetal organs and placentae for Brucella, Chlamydia, and other specific agents able to induce abortion was consistently negative, with the exception of one bovine and two ovine placentae from which fungi (*Aspergillus fumigatus* and *Mucor* spp.) grew out simultaneously. Examination of the livers and kidneys for Leptospira also gave negative results.

By *serological examination*, antibodies to *C. burnetii* were demonstrated up to the 1 : 40 dilution in serum samples taken simultaneously with abortion from three of the aborted cows. Four weeks after the first examination the sera of these dams were positive up to the same titre, or up to 1 : 20. Five of the ten other cows proved positive as well. From ewes, no positive serum was obtained by random sampling. However, the antigen prepared from pathologically-altered areas of ovine placentae, abundant in Coxiella organisms, and that prepared from the fibrinous exudate covering their surface gave a positive CF reaction with Q fever positive serum up to the dilution of 1 : 40, but did not with Brucella- and Chlamydia-positive sera.

Large masses of Coxiella were present in the spleen and lungs of guinea pigs infected with the positive inoculum and exsanguinated afterwards. The gross pathological and histopathological examination of these guinea-pigs revealed an interstitial hepatitis accompanied by infiltration by mononuclear cells, as well as a subacute, proliferative inflammation accompanied by lymphoid hyperplasia and proliferation of plasma cells in the spleen (Fig. 7). In the lungs, there was a focal, intralobular, interstitial inflammation with the presence of mononuclear cells (Fig. 8), while in the lymph nodes follicular lymphoid hyperplasia was observed.

Large numbers of Coxiella were demonstrated by microscopy in the spleen, lungs and trachea of the second-passage guinea-pigs (Fig. 9). Their gross pathological and histopathological examination revealed similar lesions as those found in animals infected with the inoculum prepared from the ovine placentae; however, this time the lung lesions were joined by perivascular and peribronchial lymphoid hyperplasia and, in some places, by intraalveolar pseudo-epithelization. No pathological lesions were found in the brain.

By electron microscopy, the pathogenic agent was found in reticulum cells of the spleen and in bronchiolar epithelial cells of the lungs of the infected experimental animals.

Bacteriological examination of organs and lymph nodes of the inoculated and exsanguinated guinea-pigs for Brucella and other specific pathogens gave negative results.

Discussion

Our present results unanimously indicate the involvement of *C. burnetii* in the aetiology of the observed abortions, and prove that Q fever associated with abortions in cattle and sheep, similarly to other countries (Babudieri, 1953; Kaplan and Bertagna 1955; Ormsbee, 1965; Thiel, 1982; Schaal, 1983), occurs also in Hungary.

Previously, other Hungarian authors (Romváry, 1957*a*, *b;* Romváry et al., 1979) had concluded to the occurrence of Q-fever-induced abortions from the seropositivity of sheep and cattle stocks; however, the pathogenic agent has not been demonstrated in Hungary so far.

The 13 cases reported by us represent the first demonstration, isolation and identification of *C. burnetii* in Hungary during the diagnostic examination of fetuses.

The organism, which was easy to observe in the light microscope, was present in large masses mostly in intracellular position, and stained red with Stamp's and Köster's stain, was demonstrated also by electron microscope in the placentae and in the spleen and lungs of experimentally-inoculated guinea-pigs. Based upon its morphology and other properties, the demonstrated pathogen was identified as *C. burnetii.*

Microscopically, the organism exhibited a pronounced pleomorphism. Coccoid forms sized 0.2 to 0.4 μm occurred together with larger, rod-shaped or sometimes thread-like, elongated forms of 0.5 to 1, or approximating even 2, μm in length within the same visual field.

Two morphologically well-distinguishable forms of *C. burnetii* have been differentiated by American and Canadian authors (McCaul and Williams, 1981; Mariassy et al., 1984; van Dreumel et al., 1984). Our present ultrastructural findings are in good agreement with the results reported by these authors.

The gross pathological and histopathological lesions seen in the fetal organs and placentae were also consistent with those described by others (Mariassy et al., 1984; Stelzner, 1984; van Dreumel et al., 1984). Also in the cases reported by us, placentitis was accompanied by vacuolar degeneration and, sometimes, diffuse necrosis of the epithelial cells of cotyledons. These lesions were directly attributable to the presence of the microorganisms.

Certain differences were noted between the placentitis occurring in sheep and that found in cattle. While in sheep mainly serous-fibrinous and, occasionally, purulent placentitis was observed, in bovine abortions mostly the haemorrhagic or necrotic lesions predominated.

Also, there was a difference between the two species as regards the lesions found in the fetal organs. In the liver and spleen of ovine fetuses, activation and, in some places, focal proliferation of the RHS cells as well as the presence of fetal haemopoietic cell colonies in a relatively large size and number were observed, accompanied by proliferation of lymphoblasts in the Malpighian bodies of the spleen and a catarrhal-purulent pneumonia. In bovine fetuses these lesions were less pronounced or completely absent, presumably because, due to the more severe placental damage, abortion had taken place within a shorter time than in sheep.

From the diagnostic point of view, the lesions observed in the fetal organs cannot be considered specific since similar changes can be produced by other abortion-inducing agents as well (Glávits et al., 1982). However, in agreement with the observations of others, vacuolar degeneration of the epithelial cells of cotyledons as well as the presence of the pathogenic agent in the cytoplasm of these cells are valuable diagnostic proofs. It seems that in the present cases *C. burnetii* exerted its pathogenic effect through its establishment and propagation in the placenta, impairing fetomaternal connection and resulting in the death of the fetus. At the same time, the lesions found in ovine fetuses indicated that the pathogenic agent had multiplied in the fetus as well.

During the laboratory diagnosis of abortions, in cattle the abortions caused by *C. burnetii* have to be differentiated from those due to brucellosis, whereas in sheep from abortions induced by *Brucella ovis* and *Chlamydia psittaci* infections.

In the present cases, Q-fever-associated abortions were of sporadic occurrence in cattle stocks; however, in sheep flocks large numbers of abortions occurred within a short time. Abortions due to Q fever were diagnosed both in the winter and in the summer period, indicating that transmission by arthropode vectors is not the only mode of transmission for *C. burnetii.* On the farms included in the study and in their vicinity we conducted on-the-spot investigations to assess the incidence of ticks. All the areas where the examined samples had come from were found to be infested by ticks. With regard to the high resistance of *C. burnetii* and to the fact that it maintains its infectivity even

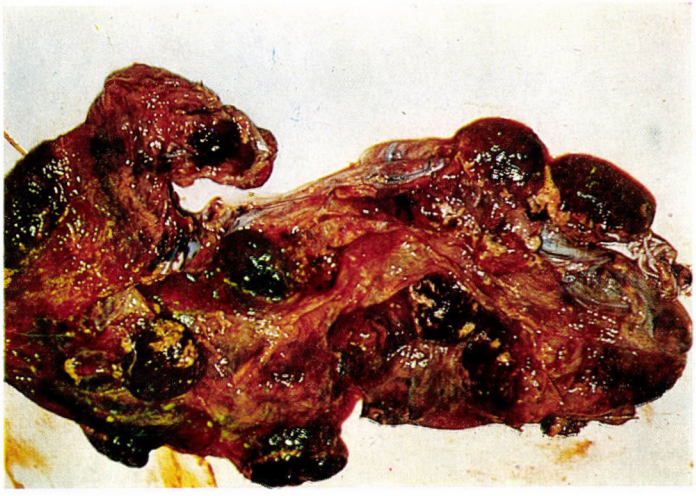

Fig. 1. Ovine placenta from *Coxiella burnetii*-induced abortion. Note the fibrinous coating on the surface of the reddened, swollen cotyledons

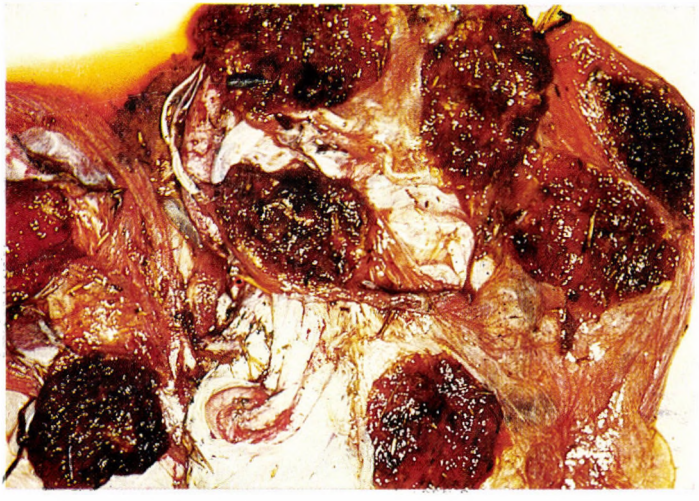

Fig. 2. Fibrinous, haemorrhagic placentitis caused by *C. burnetii* in cattle

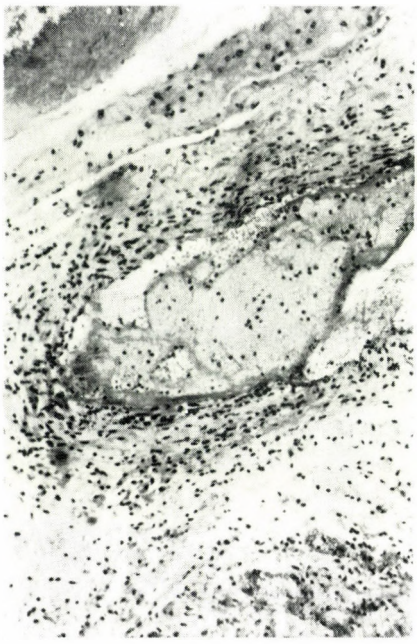

Fig. 3. Acute placentitis. Note the thrombus in the lumen of the blood vessel. H.–E., × 160

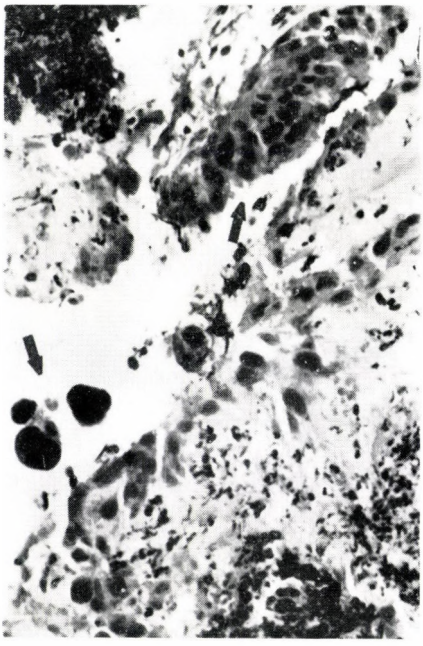

Fig. 4. Placentitis accompanied by necrosis of the epithelium of cotyledons in sheep. Masses of Coxiella (arrow) are present in most of the epithelial cells; note the clumps of Coxiella (arrow) in the detached cells. H.–E., × 400

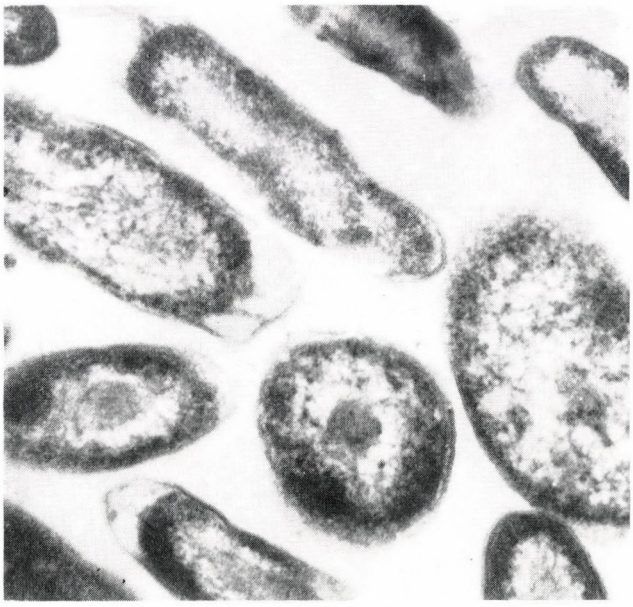

Fig. 5. Ultrastructure of *Coxiella burnetii*. Electron micrograph, × 68,000

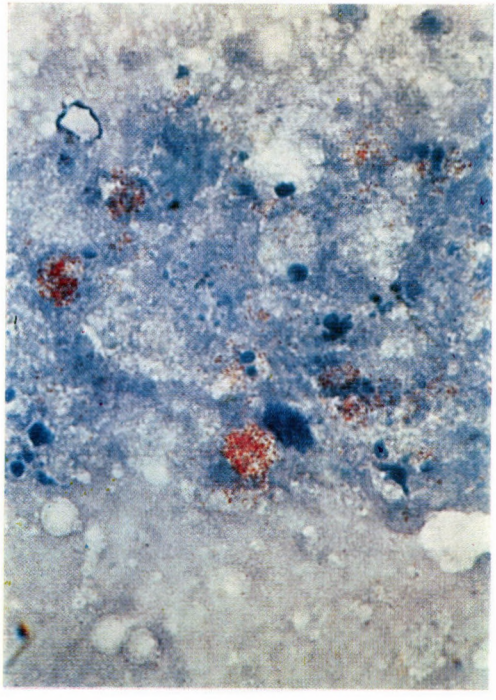

Fig. 6. Light microscopic picture of *C. burnetii* in a smear prepared from a bovine placenta. Large numbers of Coxiella fill the detached epithelial cells (arrow) and are present in the extracellular space (arrow). Stamp's stain, × 1000

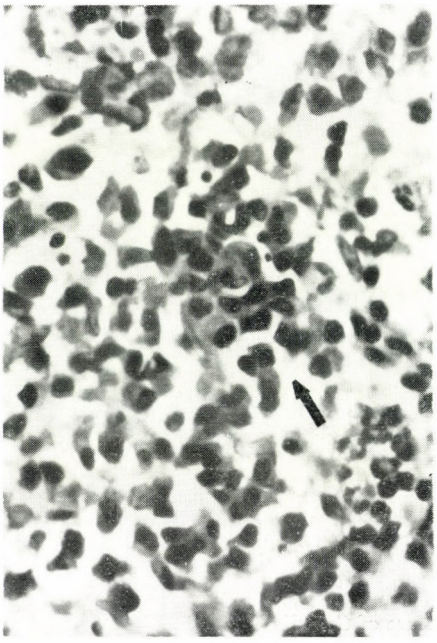

Fig. 7. Proliferation of plasma cells (arrow) in the red pulp of spleen from a guinea-pig experimentally infected with *C. burnetii.* H.–E., × 400

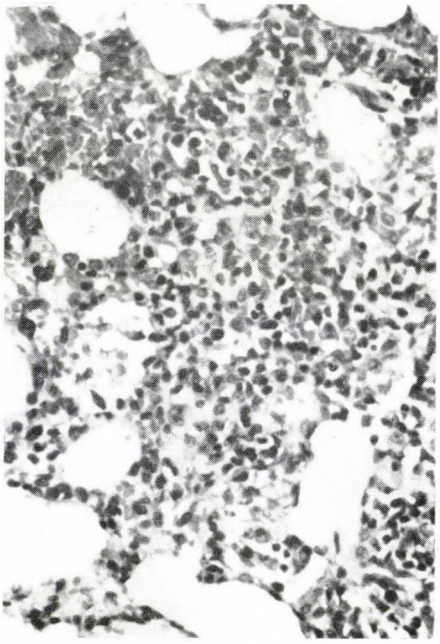

Fig. 8. Focal interstitial inflammation characterized by infiltration by mononuclear cells in the lungs from a guinea-pig experimentally infected with *C. burnetii.* H.–E., × 160

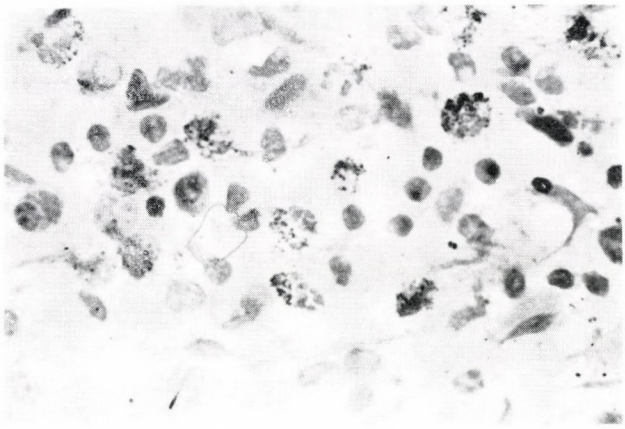

Fig. 9. Coxiella organisms in the cytoplasm of reticulum cells in a smear prepared from the spleen of an experimentally infected guinea-pig. Köster's stain. × 1000

in the dried faeces of ticks (Babudieri, 1953; Ormsbee, 1965; Thiel, 1982), a possible role of arthropode vectors cannot be excluded.

In the affected farms, economic losses due to Q fever arose from the reduced number of offspring and from the birth and subsequent death of weak calves and lambs. Total losses amount to 5 to 15%.

Man is known to be highly susceptible to *C. burnetii* (Babudieri, 1953; Farkas et al., 1950; Kaplan and Bertagna, 1955; Marmion et al., 1984; Ormsbee, 1965; Thiel, 1982). Therefore, infection of animal populations by the Q fever agent creates a potential hazard to humans, particularly to those working with animals and animal products.

In the farms where *C. burnetii*-induced abortions were demonstrated, simultaneously no human cases diagnosed as Q fever occurred. However, in other areas human cases of Q fever origin did occur (Mikola et al., 1982), indicating that the occurrence of Q fever in Hungary should be reckoned with. The highly infectious nature and great public health significance of Q fever and the economic losses inflicted point to the necessity to conduct investigations aimed at its demonstration and survey of its incidence, and to elaborate effective control measures.

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JUDGMENT OF CASTRATION-INDUCED ABSCESSES IN PIGS AT MEAT INSPECTION

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In the literature no data are available on the meat-inspectional judgment of long-existing abscesses that develop as a result of infection of the castration wound and remain unnoticed up to the time of meat inspection in normally slaughtered pigs. A total of 131 pigs with post-castration abscesses were examined. Meat pH was determined and the boiling test as well as complementary bacteriological examination of the meat were performed. From the 131 pigs pathogenic agents were isolated on 150 occasions (these included 80 isolates of *Corynebacterium pyogenes*, 52 beta-haemolysing streptococci, 7 strains belonging to the *Streptococcus viridans* group, 3 strains of each of *Staphylococcus aureus* and *Pasteurella multocida*, and 1 strain of *Salmonella choleraesuis*). From each of 66 post-castration abscesses, a single bacterium grew out in pure culture; mixed cultures of two and three bacterial strains grew out from 36 abscesses and 1 abscess, respectively. The diseased animals frequently developed bacteraemia (37 cases = 28.34%) and septicaemia (14 cases = 10.69%). The boiling test revealed sapraemia in 28 cases. Meat pH reached 6.6 or higher values in 15 cases. The final judgment was based on a combination of the above-listed partial data. The meat of only 56 pigs (42.75%) was found to be unconditionally suitable for consumption. The meat of 10 pigs (7.63%) was qualified to be of reduced nutritional value and lowered palatability due to the presence of mild abnormal odour. The meat of 50 pigs (38.16%) was judged to be conditionally suitable for consumption (conditionally admissible). Of the latter, 7 pigs (5.37%) were assigned to this category because of high meat pH, 6 pigs (4.58%) because of the contamination of the muscles with low numbers of saprophytic bacteria, whereas the remaining 37 animals (28.24%) due to bacteraemia. Fifteen pigs were judged to be unfit for human consumption; 14 of them (10.65%) because of septicaemia, and 1 (0.66%) because of extrahepatic occurrence of Salmonella. Based upon the present results it is clear that post-castration abscesses deserve more attention at the meat inspection of pig carcases.

Keywords. Castration, post-castration abscess, meat inspection, judgment, high ratio of objectionable meat, mixed infections, frequency, septicaemia, bacteraemia, sapraemia.

In recent years, meat inspection specialists have paid great attention to disease entities caused by pyogenic bacteria *(Corynebacterium pyogenes, Streptococcus* and *Staphylococcus* spp.) in normally slaughtered pigs.

Among other lesions, abscesses (Jones, 1980; Engvall and Schwann, 1983), arthritic lesions (Turner et al., 1980), decubitus ulcers (Nouws et al., 1981), and tail necroses (Van den Berg et al., 1981) have been subjected to meat-inspection judgment.

However, no references have been made in the literature to meat inspection problems associated with the abscesses occurring in normally slaughtered pigs as a consequence of wound infection following castration (hereinafter re-

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ferred to as post-castration abscesses). Therefore, we feel justified to report our observations concerning this subject.

Materials and methods

Normally slaughtered barrows with a favourable result of the previous ante-mortem examination were used. The pigs were 180 to 200 days old meat-type hybrids, with a mean slaughter weight of 95 to 100 kg. After meat inspection, individuals with post-castration abscesses were sent for laboratory examination. The final judgment was made 48 h later, when the results of laboratory examinations had already been obtained.

The complementary laboratory examination consisted of the following parts:

(1) *Determination of meat pH*, using a nitrazine yellow indicator.

(2) *Boiling test for abnormal odour* was carried out on samples taken from meat previously kept in the refrigerator for 24 h. The samples were classified into one of the following categories: negative, mild abnormal odour, strong abnormal odour.

(3) In the *complementary bacteriological meat inspection* the following media and methods were used: Of the organs of pigs, samples from the spleen, liver, kidneys, two muscles, the praescapular and the contralateral popliteal lymph nodes as well as from pathologically-altered areas were inoculated into nutrient media. Purulent exudate obtained from the post-castration abscess with a sterile cotton swab was processed in all cases.

To prepare the nutrient medium, we digested with papain and pepsin a material containing soybean extract, defibrinated bovine blood, and a 4 : 1 mixture of bovine heart and liver, and added peptone and yeast extract to the digested suspension. From the basic medium a broth of pH 7.4, agar plates containing 10% bovine blood, and a medium containing 5% bovine serum were prepared.

For the isolation of Salmonella, Preuss's broth containing potassium tetrathionate was used besides the direct inoculation of Drigalski's agar plates. Obligate anaerobic bacteria were cultured in Takács–Narayan's semi-solid medium, functioning on the principle of sulphite reduction.

Broth cultures were spread on blood agar plates in all cases; thus, both the directly inoculated blood agar plates and the subcultures on blood agar plates inoculated with the broth cultures were available for judgment.

Cases when the same pathogenic bacterium was isolated from two samples (in most cases from the post-castration abscess and from another organ) were classified as *bacteraemia*, while *septicaemia* was the diagnosis if more than two (3 to 8) samples were positive.

Results

Brief description of pathological-anatomical lesions

Castration-induced abscesses were observed in the perineal region (regio perinealis) of pigs, in a location corresponding to the cavity of the scrotum, usually on one side, less frequently on both sides, as round, elastic masses the size of a walnut or a child's head. The small abscesses were usually compact to the touch, while the large ones were occasionally slightly undulatory when palpated. Frequently the small castration abscesses embedded in the adipose tissue of the perineal region were not visible and could be observed only on the surface of the split carcase. The abscesses were usually attached to their environment; their wall consisted of a 3 to 5 mm thick, greyish-white, tough connective tissue. They contained a viscous, cream-like, yellowish or greenish, usually ill-smelling, purulent exudate frequently coagulating like the yolk of a boiled egg. In some cases not only one but two or three abscesses were found towards the internal inguinal ring. Infrequently large abscesses were accompanied by a mild scrotal and preputial oedema.

Lesions other than the castration-induced abscess occurred in 23 cases (17.56%). These included renal degeneration (4), hepatic and renal degeneration (3), dermatitis (3), swelling of the spleen (2), renal haemorrhages (1), pulmonary abscess (1), bronchopneumonia (2), purulent nephritis (1), paronychia (1), and slightly imperfect bleeding (1).

Complementary bacteriological meat inspection

During the 15-month period between 1 January 1983 and 31 March 1984, out of 545,798 normally slaughtered pigs 131 (0.024%) were found to have castration-induced abscesses.

During the period of study, 1300 pigs (0.24% of the slaughtered pigs) were subjected, for different reasons, to complementary bacteriological meat inspection. The 131 castration-induced abscesses constituted 10.07% of the pigs sent for complementary bacteriological meat inspection.

Exudate present in the abscesses was examined on 113 occasions altogether; in 18 cases (13.74%) the exudate was not examined due to sampling problems. Ten swabs (8.84% of the swabs examined) yielded no pathogenic bacteria on culturing.

Of the 131 pigs with castration-induced abscesses, pathogenic bacteria belonging to a total of seven species or groups were isolated on 150 occasions (Table I). Most of them were *Corynebacterium pyogenes* and beta-haemolysing streptococci, while the remaining bacteria were members of the *Streptococcus viridans* group, *Staphylococcus hyicus*, *Staphylococcus aureus*, *Pasteurella multocida* and *Salmonella choleraesuis*.

Table I

Type, number and percentile proportion of bacterial strains among the 150 isolates cultured from the 131 pigs examined because of castration-induced abscesses

Bacterial strain	Number	%
Corynebacterium pyogenes	80	53.33
Beta-haemolysing streptococci	52	34.66
Streptococcus viridans	7	4.66
Staphylococcus hyicus	4	2.66
Staphylococcus aureus	3	2.00
Pasteurella multocida	3	2.00
Salmonella choleraesuis	1	0.66
Total	150	

The castrational abscesses of 66 pigs (58.4%) yielded pathogenic bacteria in pure culture; those of 36 pigs (31.85%) yielded a mixed culture consisting of two bacteria, while from one pig (0.88%) a mixed culture of three bacteria was obtained (Table II).

Acting as a focus of infection, the castration-induced abscess resulted in the development of bacteraemia in 37 pigs, while in 14 pigs septicaemia occurred. Of these, 6 cases of bacteraemia and one case of septicaemia were observed in pigs whose abscess was not examined bacteriologically.

With a few exceptions, during bacteraemia and septicaemia starting from the mixed microflora of the castration-induced abscesses not all but usually only one component of the bacterial flora, in most cases streptococci, disseminated in the organism.

Meat-inspectional judgment of pigs with castration-induced abscesses

The results of meat inspection of the 1321 pigs examined because of castration-induced abscesses are shown in Table III.

The meat of 56 pigs (42.75%) was considered to be unconditionally suitable for consumption.

The meat of 10 animals (7.63%) proved to be of reduced nutritional value and palatability due to the presence of a mild abnormal odour of outside source (an odour resembling the smell of carious teeth, urine, sexual odour, or the combination of these). These meats were sold in fresh state in official butcher's shops.

The meat of 50 pigs (38.16%) was judged to be conditionally admissible, partly because of the low meat pH due to abnormal ripening, partly because of the presence of low numbers of saprophytic bacteria (micrococci, members of the Enterobacteriaceae family, or aerobic spore-forming bacteria of the *Bacillus* genus) which occurred as a result of the animals' impaired resistance, or because of bacteraemia caused by pathogenic bacteria. These meats were

Table II

Bacteria present in the castrational abscesses and those involved in bacteraemia and septicaemia starting from the abscesses

	Bacterial content in the exudate of the abscess	Number of cases	Bacteraemia starting from the abscess		Septicaemia starting from the abscess	
	Not examined	18	*C. pyogenes*	2	*C. pyogenes*	1
			Beta-haemolysing str.	2		
			Beta-haemolysing str. + *Staph. hyicus*	1		
			Beta-haemolysing str. + *Str. viridans*	1		
	Saprophytic bacteria	10				
One pathogen was present in the abscess	*Corynebacterium pyogenes*	43	*C. pyogenes*	10	*C. pyogenes*	3
	Beta-haemolysing str.	15	Beta-haem. s.	7	Beta-haem. s.	4
	Streptococcus viridans	2	*Str. viridans*	1		
	Staphylococcus hyicus	1				
	Staphylococcus aureus	2	*Staph. aureus*	1		
	Pasteurella multocida	2				
	Salmonella choleraesuis	1				
Two pathogens were present in the abscess	*C. pyogenes* + beta-haem. str.	29	Beta-haem. s.	5	Beta-haem. s.	4
	C. pyogenes + *Str. viridans*	3	*Str. viridans*	1		
	C. pyogenes + *St. aureus*	1	*Staph. aureus*	1		
	Beta-haem. str. + *Str. viridans*	1	Beta-haem. s.	1		
	Beta-haem. str. + *Pasteurella multocida*	1				
	Beta-haem. str. + *Staphylococcus hyicus*	1			*Staph. hyicus*	1
Three pathogens were present in the abscess	Beta-haemolysing streptococci + *C. pyogenes* + *Staph. hyicus*	1			*Staph. hyicus*	1
	Total	131		37		14

sold in official butcher's shops after heat treatment by boiling. It should be mentioned that although pH values of 6.6 or higher occurred on 15 occasions, only in 7 cases did the adverse pH value in itself act as the sole cause of regarding the meat as conditionally admissible.

The meat of 15 animals (11.45%) was judged to be unfit for consumption. In 14 pigs septicaemia caused by pathogenic bacteria, while in one animal the occurrence of *Salmonella choleraesuis* outside the liver (in the exudate of the castration-induced abscess) was observed.

Table III

Meat-inspectional judgment of the 131 pigs examined because of castration-induced abscesses

Judgment	n	%	Positive boiling test	High pH	Pathological lesions in other organs
Unconditionally suitable for consumption	56	42.75	—	—	7
Reduced nutritional value and palatability because of mild abnormal odour	10	7.63	10	—	2
Conditionally admissible					
(1) High pH (above 6.6)	7	5.34	2	7	1
(2) Viscera and muscles contaminated with low numbers of saprophytic bacteria	6	4.58	4	1	2
(3) Bacteraemia	37	28.24	9	6	7
Unfit for consumption					
(1) Septicaemia	14	10.68	3	1	4
(2) Extrahepatic occurrence of Salmonella	1	0.76	—	—	—
Total	131	99.98	28	15	23

Discussion

In pigs, the most frequent surgical intervention is emasculation of male animals (castration). Although the operation is simple, complications are not infrequent. These include haemorrhage, prolapse of the intestine and omentum, accumulation of clotted blood or serum, abscess formation, purulent funiculitis, suppuration of the inguinal lymph node, tetanus, and cyst of the vaginal membrane (Tamás and Fellner, 1960). Of the above-listed complications, the castration-induced abscess developing as a result of wound infection is one of the most frequent and significant lesions (Schuster, 1982); this is the lesion most frequently encountered also in normally slaughtered pigs.

Optimally, castration should be performed on piglets; however, for different reasons, many pigs are castrated at 3 to 4, or, in farms producing breeding animals, as late as at 6 months of age. Delayed castration may furnish an explanation for the high frequency of castrational abscesses at slaughter.

It is remarkable and surprising that, based upon the laboratory examinations, only the meat of 42.75% of the pigs with castration-induced abscesses proved to be unconditionally suitable for consumption, while 57.25% of the examined animals had to be withheld from unconditional release in some way or other. This high ratio of objectionable meat indicates that in the future more

attention should be paid to this lesion, which has usually been neglected so far from the viewpoint of meat inspection.

The disinterestedness displayed so far by the meat inspection service in castration-induced abscesses is shown by the fact that no data are available in the literature on the meat-inspectional bearings of such abscesses.

In the present studies, the high incidence (38.93%) of bacteraemia (37 pigs) and septicaemia (14 pigs) starting from the castrational abscess, and caused by bacteria present in the abscess, was conspicuous. Most of the pathogens were *Corynebacterium pyogenes* or beta-haemolysing streptococci. The above facts prove that bacteria present in a long-existing, circumscribed, apparently inactive, encapsulated focus like a castration-induced abscess frequently invade the organism, as a result of impaired resistance primarily associated with an increased stress during transportation. The risk constituted by such encapsulated abscesses was pointed out earlier (Forray and Százados, 1969), in association with the disease caused in cattle by foreign bodies.

Bacterial dissemination after castration was reported, although not from the viewpoint of meat inspection, by Ramisse et al. (1978). These authors observed nervous symptoms in a farm producing 1500 to 1800 piglets, after castration. The morbidity was 15%, the mortality 5%. A group-K streptococcus was isolated from the haemorrhagic meningitis diagnosed at necropsy.

Examination of the exudate present in the castration-induced abscess is of decisive importance during the complementary bacteriological meat inspection. This is shown by cases of bacteraemia which could be diagnosed in the absence of the affected organ; however, if the affected organ had been examined, these cases should have been judged more strictly. In cases of septicaemia, the number of positive organs within an animal was low; in all cases but one the same pathogen was isolated simultaneously from at most three organs of a pig. The only exception was a case of septicaemia caused by a beta-haemolysing streptococcus; however, here also only four samples were positive of the eight samples tested. Thus, castrational abscesses of pigs are characterized by a numerically frequent, but as regards dissemination within the organism only a moderate, bacterial invasion.

The boiling test has great importance in the judgment of castration-induced abscesses. In the present study, positive boiling test occurred frequently. In most cases the nature of the odour was indicative of sapraemia. The boiling test gave positive results in 28 cases (21.37%); however, in only 10 carcases (7.63%) did it serve as the basis of judgment.

The measurement of meat pH is a useful complement to the laboratory examinations. Although meat pH values of 6.6 or higher were found on 15 occasions, only in 7 pigs did meat pH serve as the sole basis of judgment (similarly to the result of the boiling test), since more than a half of the cases were

characterized by adverse bacteriological results which served as the primary basis of judgment.

Other pathological lesions accompanying the castration-induced abscess are hard to recognize. Although such lesions might be mild or might not be related to the abscess at all, in most instances they occur in cases with unfavourable meat-inspectional judgment; therefore, they deserve particular attention.

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IN VITRO DEMONSTRATION OF THE CONVERSION OF THYROXINE TO 3,3',5-TRIIODOTHYRONINE IN LIVER BIOPSY SAMPLES TAKEN FROM LACTATING COWS

(SHORT COMMUNICATION)

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Conversion of thyroxine to triiodothyronine was studied on 20 liver biopsy samples taken from lactating cows. In an in vitro system 0.61 μM thyroxine was added to the liver homogenate prepared from 200 to 500 mg bioptate.

The rate of liver 5'-monodeiodination was 28.72 $\pm$ 3.95 pmol triiodothyronine/l g fresh tissue/h or 38.16 $\pm$ 6.15 pmol triiodothyronine/100 mg protein/h.

The present paper demonstrates the existence of conversion of thyroxine to triiodothyronine in the liver of cattle.

Keywords. Thyroxine, triiodothyronine, conversion, liver, cattle, in vitro.

Thyroid hormones are of special interest in the case of lactating cows because of the unique energy balance occurring around parturition. Several papers have dealt with the relation of thyroid economy and energy utilization of the cow (Hart et al., 1978; Trenkle, 1978; Kunz and Blum, 1981; Roberts, 1982; Pethes et al., 1985) and with the environmental relations of the thyroid function in ruminants (Osmond et al., 1981; Magdub et al., 1982; Valtorta et al., 1981). The method applied in these studies to establish thyroid function is the measurement of thyroid hormones (mainly thyroxine and 3,3',5-triiodothyronine) by specific radioimmunoassays. In spite of the expectations, results did not clearly demonstrate the supposed regulatory role of thyroid hormones in the above cases. One should assume that one possible reason for this might be the approach applied. Namely, it has been proved that the actual level of thyroid hormones in the circulation will not reflect the momentary hormone supply at the cellular level (for review, see Larsen et al., 1981). In the background of this phenomenon one will find the intricate mechanism of activation and inactivation of thyroxine arriving to the tissues via the circulation: thyroxine (T4) is 5'-monodeiodinated into 3,3',5-triiodothyronine (T3) and in this way activated, i.e. T3 will bind to the intracellular receptors and exert the biological effect. The other way round, when 5-monodeiodination occurs, the biologically inactive metabolite, 3,3',5'-triiodothyronine (reverse-T3, r-T3) will be produced (see for review: Cavalieri, 1980). It is likely that the major point of the regulation is the adjustment of the activity of the 5'-monodeiodinase by both the hormone metabolites themselves and other intracellular factors. Since this re-

gulatory pathway could be demonstrated in several mammalian species (Kaplan, 1983) and also in birds (Kühn et al., 1984), and since direct evidence of the presence of r-T3 in the serum of the cow has been shown (Rudas, 1980; Pethes et al., 1985), one can suggest that it holds true of cattle, too.

The aim of the present communication is to show the existence of T4 to T3 conversion in cattle and to provide a method by which the rate of deiodination can be established in the living animal in order to obtain a better parameter for estimating the momentary thyroid status of the cow.

In all the cases described up to the present, the liver proved to be the major site of monodeiodination; in addition, this is the organ one can take biopsy samples of with relative ease. Therefore, liver biopsy was carried out on 20 Holstein-Friesian dairy cows according to the method described by Gaál and Husvéth (1982). Briefly, it is a modified aspiration biopsy technique under local anaesthesia. The animals were selected randomly on a dairy farm and sampled after parturition, within ten days. Biopsy samples were transported to the laboratory, deep-frozen and kept below −20 °C until used.

The activity of the deiodinase was measured according to the method published by Kaplan (1980). The modification was described in detail in a previous paper (Scanes et al., 1983): 200 to 500 mg of liver is weighed and transferred into plastic tubes (75 mm × 7 mm in diameter) containing sufficient amount of 0.15 M potassium phosphate buffer (pH 7.4) to give a final buffer to liver ratio of 3 : 1 (v/w). After homogenization, incubation was carried out with aliquots of the sample. Control tubes for original T3 contamination, initial T3 production and for conversion of 0.61 μM T4 in the presence and absence of dithiothreitol (DTT, Sigma Chemical Co., St. Louis) were run simultaneously in an incubation system for 3 h, at 37 °C. The T3 contents of the alcoholic extracts of the supernatants were determined by a radioimmunoassay (RIA) system developed earlier (Pethes et al., 1978). Protein was measured according to Lowry et al. (1951). The results are expressed as pmol T3 produced under DTT stimulation per gram fresh liver or per 100 mg of protein.

Table I summarizes the results obtained by measuring serum levels of thyroxine, triiodothyronine and liver deiodinase activity. Normal values of T4 and T3 obtained here do not differ from that found by others (Kunz and Blum, 1981) or by us earlier (Pethes et al., 1978). The activity of monodeiodinase can only be compared to results obtained in other species. In rats one can find 15–20 pmol T3/g liver/h (Kaplan and Utiger, 1978), what is slightly lower, and in fowl 27–34 pmol T3/g/h values, what are slightly higher than that found here for the lactating cow (28.72 $\pm$ 3.95). Despite the minor differences one can conclude that no major species difference exists as for the activity of the monodeiodinase enzyme. This does not mean that further characterization of the enzyme (i.e. determination of its V_{max}, homogeneity and relative intracellular quantity) will not reveal species differences.

Table I

Serum T4 and T3 values and the activity of the monodeiodinase enzyme in liver biopsy samples from lactating cows ($\bar{x} \pm$ SEM (n))

Serum		Liver 5'-deiodinase activity pmol T3 produced/h by	
T4	T3		
(ng/ml)		1 g fresh tissue	100 mg protein
65.48±8.95 (20)	1.34±0.2 (20)	28.72±3.95 (20)	38.16±6.15 (20)

As it was mentioned before, the usefulness of this method is demonstrated in another paper (Pethes et al., 1985) where it was applied to follow up the effects of different nutritional statuses on thyroid economy.

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SEASONAL CHANGES IN THE PLASMA CONCENTRATION OF SEXUAL STEROIDS, CORTICOSTERONE AND THYROID HORMONE IN THE HEN WITH SPECIAL RESPECT TO THE MOULTING PERIOD

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The seasonal endocrine rhythms of the domestic hen *(Gallus domesticus)* were studied under natural circumstances in the temperate zone. Plasma concentration of progesterone increased at the beginning of the egg-laying period and in May, culminated during the time of maximal egg production, while in summer it decreased significantly before the subsequent increase at the end of summer, concurrently with the peak of moulting. The maximum level of oestrone is present at the time of seasonal maturation (right before the egg-laying period); after its decrease several minor peaks appear during the time of maximum egg production and at the end of summer. Testosterone peaks at the time of maximum egg production and at the end of summer, quite similarly to oestrone. The level of 17β-oestradiol does not produce significant seasonal changes.

Apart from the progesterone peak, sexual steroids show low levels at the time of moulting. The plasma level of corticosterone increases parallel to the lengthening illumination during seasonal maturation and at the beginning of the egg-laying period. There is a drop in April, followed by the peak hormone concentrations in the period of maximum egg production. The corticosterone level decreases in summer and shows a minor peak at the time of moulting.

Peak thyroxine values occur in the cold winter period and later around the egg-producing period. Maximum thyroxine concentrations can be measured some weeks before moulting. The level of triiodothyronine is high in winter and reaches its maximum during the weeks following moulting.

Keywords. *Gallus domesticus*, sexual steroids, corticosterone, thyroid hormones, moulting period.

Due to the modern analytical methods (competitive protein binding assay (CPBA), radioimmunoassay (RIA)) for hormone determinations one can find a considerable amount of papers dealing with the seasonal endocrine cycles of different avian species. Data have been accumulated concerning the duck (Assenmacher et al., 1975; Haase and Paulke, 1980; Haase and Sharp, 1975; Jallageas et al., 1978), the white-crowned sparrow *(Zonotrichia leucophrys gambelii*, Smith, 1979; Wingfield and Farner, 1978*a*, *b)*, the starling (Temple, 1974), the wild turkey (Burke et al., 1977), the Canada goose (John and George, 1978), the ruffed grouse (Garbutt et al., 1979), the collared dove (Péczely and Pethes, 1979, 1980), the rook (Lincoln et al., 1980; Péczely and Pethes, 1982) and the spotted munia (Chandola et al., 1980).

The favourite species for endocrine studies in birds is *Gallus domesticus*. Many-sided experiments have been carried out on this species in different environmental circumstances (lighting, temperature, feeding). However, we

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failed to find data concerning seasonal changes of *Gallus domesticus* kept under natural circumstances. Besides the aim of filling this gap, another reason was considered while undertaking the present work. Particular stress was laid on examining the natural moulting in autumn since investigating this period is a continuation of our former experiments (Pethes et al., 1982, 1983; Szelényi et al., 1981, 1983), dealing with moulting in artificial surroundings.

Materials and methods

The experiment was begun in January 1982 in Budapest. Eight 27-week-old New Hampshire hens (among them 7 layers) and 1 rooster were used. This favourite type of household farming was placed 8 weeks before the beginning of the experiment on spot. A hen-run of 20 m^2 was provided beside the hen-house. Without using any light or heating effect, the birds were exposed to the extremes of the temperate climate. As for feeding, we tried to be traditionalists. Besides the layer feed produced by a feed-plant, the birds were given kitchen and garden waste. Water was provided ad libitum. At the time of the monthly blood sampling a complete vitamin supplement was also given (Phylasol combi, Phylaxia, Budapest). In spite of being 27 weeks old or more at the beginning of the experiments, the birds did not lay eggs. The rather cold winter might serve as a reason for this phenomenon.

Parallel to the warming up, at the end of February the egg-laying period began. Egg production was maximal in May, then it decreased gradually. The fall moulting began at the beginning of October. After moulting, egg production of a low level could be observed, stopped by the severe winter colds in December.

Blood samples were taken monthly (on the first day of the month) and an additional sample was taken on October 15. The blood samples were centrifuged at 3000 rpm, the plasma was kept below −20 °C until used. Plasma concentrations of the sexual steroids (progesterone (PROG), testosterone (TEST), oestrone (E_1), 17-β-oestradiol (E_2)) were determined after separation on Sephadex LH-20 by subsequent radioimmunoassay (RIA) as described by Péczely et al. (1980*b*). Thyroxine (T4) and triiodothyronine (T3) were determined by direct RIA (Pethes et al., 1978). Corticosterone (CRT) was measured by CPBA according to Murphy (1967).

Results

Sexual steroids (Fig. 1)

PROG was rising from the beginning of the ovulatory cycle until May, when, at the time of most intensive egg-laying, it showed an annual peak. In

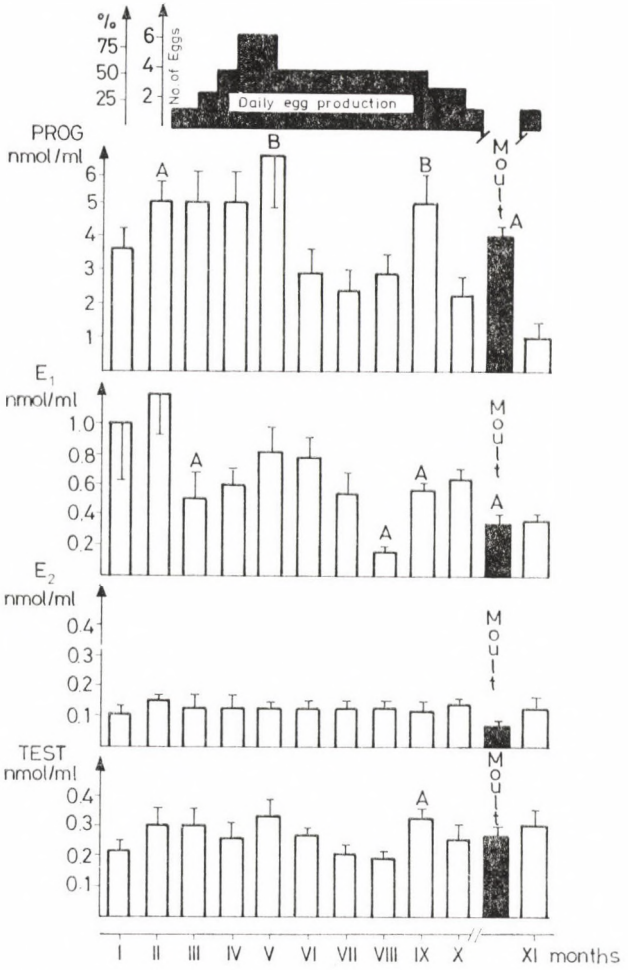

Fig. 1. Seasonal changes in sexual steroid levels (progesterone, PROG; oestrone, E_1; 17β-oestradiol, E_2; testosterone, TEST). A = $P < 0.001$; B = $P < 0.05$ (compared with the previous month)

summer, it significantly decreased, then it rose again in September. There was a small but significant peak at the time of moulting. The two peaks of TEST (in May and September) parallel to those found in the case of PROG refer to the intensive metabolic stage of egg-laying. E_1 showed an annual peak at the time of seasonal maturation in February, reached a plateau during the egg-producing period in May and showed a similar tendency to PROG and TEST in September. No significant fluctuation of E_2 could be detected around the year. Oestrogen and TEST levels in the plasma were low in the moulting period. The peak plasma level of PROG at this period refers to its important physiological implications in moulting.

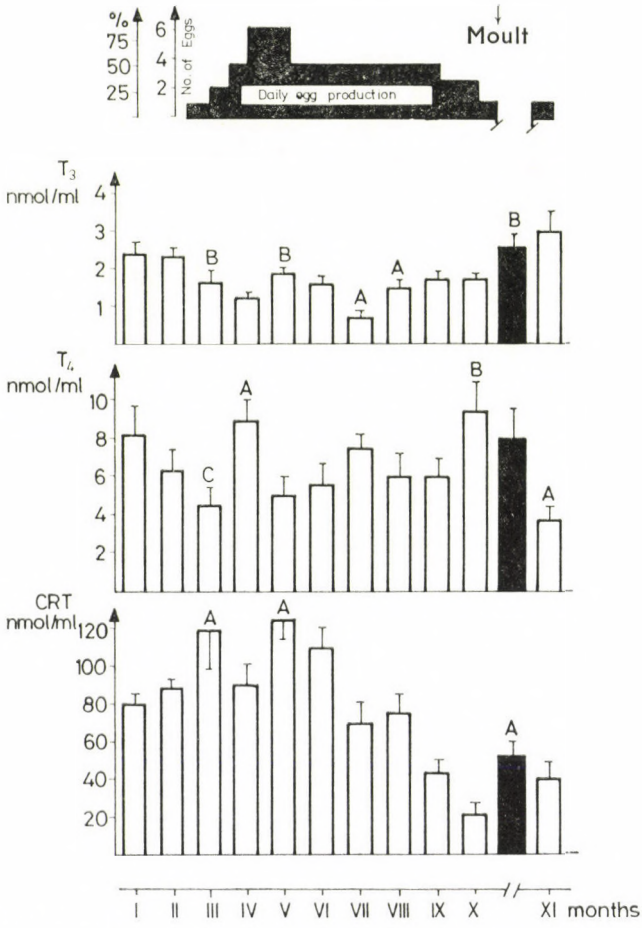

Fig. 2. Seasonal changes in triiodothyronine (T3), thyroxine (T4) and corticosterone (CRT) levels. A = $P < 0.001$; B = $P < 0.05$ (compared with the previous month); C = $0.01 > P < 0.05$ (compared with data received in January)

Thyroid hormones (Fig. 2)

The high plasma concentration of T4 in January decreased gradually after the cold period. It exhibited a significant peak when egg-laying became more intensive in April. After a non-significant fluctuation it reached its annual peak just before the fall moulting in October and a plateau during moulting. T3 level dropped in early spring, just like T4. High T3 concentrations can be seen at the time of the intensive egg-producing period in May. Its plasma concentration decreased in summer, after which there was a gradual elevation until the annual peak during the weeks past moulting in November.

Corticosterone (Fig. 2)

The elevation of CRT plasma concentration at the beginning of the year was in connection with the increased lighting and enhanced ovarian activity. A significant trough could be detected at the time of maximum egg production in May, referring to the synergism with PROG. In summer there was a drop in the plasma concentration of CRT and there were minimum values in October. At the time of moulting a minor but significant peak was observed.

Discussion

The elevation of plasma PROG is closely related to the beginning of egg-laying. Also, the maximum of egg production is accompanied by a peak of the PROG level. This agrees with the well-known fact that in the hen ovulation is preceded by a preovulatory PROG surge (Furr et al., 1973; Laquë et al., 1975; Shababi et al., 1975). On the basis of data published in the literature and of our own observations (Péczely et al., 1980*b*) we also consider the sudden release of PROG as a trigger of the preovulatory LH peak.

The two seasonal troughs in the case of TEST parallel those of the PROG curve. Both regular (Petterson et al., 1973; Shababi et al., 1975) and irregular (Péczely et al., 1980*b*; Shababi et al., 1975) fluctuations of the TEST level are known during the ovulatory cycle of the hen. In our opinion the changes in TEST concentration are in connection with the increased metabolic activities during egg production rather than with being a stimulus for LH release, as stated by others (Etches and Cunningham, 1977). In favour of the former supposition is the fact that anti-androgen treatment cannot stop the normal ovulation in the hen (Luck, 1982). It is known that the maximal size of the gonads parallels the highest TEST level in *Zonotrichia* species. In this case, however, the high level of TEST is proved to be in connection with courting, defence of territory and with nest-building activities, and not with ovulation.

The peak concentration of E_1 at the beginning of the year agrees well with the findings of Senior (1974) who detected a maximum level of oestrogens at the beginning of egg-laying. The high concentrations of oestrogens in the plasma of the hen are responsible for the physiological preparation of the hen for egg-laying. First of all, these hormones are involved in the development of the Ca^{2+} storage of the medullary bones and in the maturation of the oviduct. It has been suggested (Petterson and Common, 1973) that regular oestrogen peaks are characteristic of the ovulatory cycle in the hen (Petterson and Common, 1973), but this could not be substantiated later (Laquë et al., 1975; Kühn and Nouwen, 1978; Senior, 1974). The maximum levels of E_1 and E_2 precede the maximum size of the ovary also in other species, e.g. *Zonotrichia leucophrys gambelii* (Wingfield and Farner, 1978*b*), where peak levels of these

hormones occur during courting, mating and nest-building. In *Zonotrichia leucophrys pugetensis* (Wingfield and Farner, 1978*a*) the maximal annual level of the oestrogens coincides with oviposition. In the rook, too, a peak E_2 level can be found at oviposition (Péczely and Pethes, 1982). In the collared dove there is a maximum of E_1 concentration during incubation, and a peak of the E_2 curve during active ovarian function (large follicles; Péczely and Pethes, 1979).

The lack of rhythmic changes in the E_2 level in hens suggests that E_2 plays a less important role in the sexually mature hen relative to E_1 (Petterson and Common, 1973; Senior, 1974). This is supported by our previous experiments in which not only higher levels of E_1 were measured, but also the fluctuations in the plasma concentration of E_1 coincided with the physiological variations, unlike the changes in the E_2 level. The more important role of E_1 in sexually mature birds is also stressed by other findings (Péczely and Pethes, 1982; Wingfield and Farner, 1978*a*).

Oestrogens and TEST exhibit low levels during moulting. The same applies to other species, too (Péczely and Pethes, 1979, 1982; Wingfield and Farner, 1978*a*, *b*). A moult-ceasing effect of high levels of androgens and oestrogens is also known (Farner and King, 1972). Forced moult could not be initiated by treatment with TEST (Juhn and Harris, 1956; Kobayashi, 1958) or with oestrogens (Kobayashi, 1958).

Results obtained by others (Adams, 1956; Harris and Shaffner, 1957; Juhn and Harris, 1956; Kobayashi, 1958) and by our team (Pethes et al., 1982; Szelényi et al., 1981, 1983) have proved the importance of PROG in the initiation of moulting under artificial experimental circumstances. The present results add a new aspect to the former findings; namely, that the significantly high level of PROG around moulting occurs also in natural environment. In two cases when the PROG level was measured by means of RIA, there was no evidence of elevated hormone concentrations in the moulting hen (Furr, 1973; Gildersleeve et al., 1982). The early phase of the forced moult was investigated by Gildersleeve et al. (1982). In the corresponding phase of the moulting period we, too, failed to demonstrate the elevation of plasma PROG; an elevation was detectable only later (Szelényi et al., 1981; Pethes et al., 1982). Furr (1973) did not investigate the entire period of moulting, and one can suppose that the sampling coincided with the low PROG plasma concentration.

The activity of the thyroid gland increases during winter (Hendrich and Turner, 1967; Stahl and Turner, 1961), and it is also well known that cold activates the thyroids (Kühn and Nouwen, 1978). This might be the reason for the decrease in the plasma concentration of thyroid hormones observed when the average daily temperature begins to rise. The elevation of thyroid hormone levels in spring is preceded by the PROG peak. PROG treatment, unlike TEST and oestrogens, was a successful means in enhancing the T4 level in ovariecto-

mized Japanese quail (Pethes and Péczely, 1981). On the contrary, the level of T4 decreases in continuously laying, sexually mature Japanese quail; later, while PROG is at the maximum level, one can observe low T4 concentrations. This was the case in the present experiment, too. It is of interest that the level of the sexual steroids is low when the annual peak of the T3 curve is present in July.

The annual peak of the plasma concentration of T4 can be demonstrated before the annual moult in fall. A similar phenomenon can be observed in other species, the duck (Assenmacher et al., 1975) and the rook (Péczely and Pethes, 1982). There is a similarity between the rook and the sparrow in that both T4 and T3 peaks parallel at the post-nuptial moult (Lincoln et al., 1980; Smith, 1979). T4 behaves similarly in the wild turkey (Burke et al., 1977) and in *Zonotrichia leucophrys gambelii* (Smith, 1979) around post-nuptial moult, but there is no pre-nuptial T4 peak in that species. According to the present results, T3 is elevating parallel to T4, but it reaches its maximum later. From the point of view of moulting the T3 peak coincides with the fledging. Similar results were found in the case of two wild species. In mallards there is a phase shift of the maximum levels of T3 and T4 (Haase and Paulke, 1980). In the ruffed grouse this phase shift is present during intensive moulting, and thyroid hormones exhibit peak values; however, this concentration is lower than the annual peak (Garbutt et al., 1979). The importance of thyroid hormones in the moult has been suggested in earlier works (Gildersleeve et al., 1982). The results outlined above and obtained from experiments with forced moult (Brake et al., 1979; Pethes et al., 1982; Szelényi et al., 1981) are contradictory. In variance with Brake et al. (1979) we suppose that the initiation of moulting is bound to T4, and T3 is important only in the subsequent phase, namely while fledging. At the beginning of moult, however, T4 does not act alone, but it exerts its effect together with PROG.

According to the literature (Brake et al., 1979; Gildersleeve et al., 1982; Pethes et al., 1982; Szelényi et al., 1981), moult is accompanied by ovarian regression (no ovulation and oviposition). This gives rise to the supposition that the PROG appearing at that time is of adrenal origin. In his classical experiment Kobayashi (1958) provoked moult by giving T4 to either gonadectomized or intact birds.

Changes in the plasma concentration of CRT are closely related to the lighting regime. In wild birds, the CRT level increases with increasing illumination (Wingfield and Farner, 1978*b*). At the beginning of the year the increasing illumination elevates plasma CRT. T4, PROG, and oestrogens are stimulating CRT in laboratory circumstances in Japanese quail (Péczely et al., 1980*a*). This might also be a reason of the elevation of CRT at the beginning of the year and also of the coincidence of the annual peaks of CRT and PROG. An elevation of CRT level was also detected in *Zonotrichia* species during the breeding season (Wingfield and Farner, 1978*a*, *b*).

The PROG and CRT levels are high during moult also in the hen. Similar postnuptial elevation of hormone concentrations can be observed, of the wild species, only in rooks (Péczely and Pethes, 1982; Wingfield and Farner, 1978*a*, *b*). The CRT level is high in some European warbler species as demonstrated in in vitro experiments (Péczely, 1976); however, the maximum hormone concentration was measured during migration.

In spite of early findings (Perek and Eckstein, 1959), the importance of adrenal steroids in the process of moult could not be confirmed recently (Brake et al., 1979; Gildersleeve et al., 1982; Pethes et al., 1983). Comparison of results is difficult since forced moult was used in all the above experiments. It is hard to be right in our judgement whether or not the applied environmental factors (temperature, lighting, fast) influenced the results.

There are contradictory results in connection with exogenous CRT treatment, too. Some authors succeeded in inducing moult in this way (Juhn and Harris, 1956), others failed to do so (Kobayashi, 1958). Results accumulated so far speak in favour of no role of CRT during the period of feather loss.

A comparison of the present results with those of others underline the necessity of complex evaluation. It should be considered that the phylogenetic state of a particular species might be of primary importance in forming hormonal interrelationships. Such a complex process is moulting itself. On the basis of the results obtained in this laboratory so far, we conclude that moulting is influenced by an interaction of thyroid and gonads. Further investigations are needed for an exact clarification and explanation of the details of moulting and also of the origin of plasma PROG.

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INFLUENCE OF THE MODE AND TIMING OF CALVING ASSISTANCE ON THE ACID–BASE BALANCE OF DAM AND NEWBORN CALF

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The acid–base balance of the healthy parturient dam is markedly stable, whereas that of her calf depends primarily on the length of time elapsing until calving assistance, and on the mode of assistance.

Belated calving assistance accounts for a shift of the maternal acid–base balance towards metabolic acidosis only in those cases in which difficult calving is associated with continuous strain, and exhaustion leads to recumbency of the dam. In such cases the calf is either born in a state of severe asphyxia, or dies *in utero*, owing to long-standing uteroplacentar circulation insufficiency consequent upon delay of assistance.

If the dam becomes diseased immediately before calving and makes a slow recovery, death of the fetus can be prevented by Caesarean section.

Keywords. Calving assistance, mode, timing, acid–base balance, newborn calf, cow.

Pioneer investigations into the acid–base balance of the newborn calf (Ammann et al., 1974; Moore, 1969; Mülling et al., 1972) were followed within a few years by extensive studies along the same line on calves born with or without calving assistance. Only few authors have, however, studied the acid–base balance of the parturient dam and newborn calf simultaneously, during or immediately after calving. Eichler-Steinhauff (1977) determined the blood pH, pCO_2 and pO_2 of clinically healthy parturient dams, and Held (1983) measured the blood pH, pCO_2, base excess (BE) and standard bicarbonate (SBC) of parturient dams before receiving assistance: neither author made reference to the course of parturition and mode of assistance rendered. Held (1983) also determined the acid–base parameters of diseased cows. Moore (1969) measured the blood pH, pCO_2 and actual bicarbonate (HCO_3^-) of dams within 10 min after spontaneous calving, whereas Mülling et al. (1972) compared the blood pH values after unassisted calving and assistance by moderate traction, and Ammann et al. (1974) compared the blood pH, pCO_2 and SBC values after Caesarean section. Other workers (Maurer-Schweizer et al., 1977; Schlerka et al., 1979) compared the values of the acid–base parameters of newborn calves to those of healthy non-pregnant cows for reference. For comparison, the physiological values of the acid–base parameters of clinically healthy, non-pregnant cows are the following (Schotman, 1971): pH = = 7.325—7.45; pCO_2 = 35—53 mm Hg (4.7—7.0 kPa); BE = —3.5—3.5 mmol/l; SBC = 21—27 mmol/l. The mean values given for these parameters

by Maurer-Schweizer et al. (1977) and Schlerka et al. (1979) fall into the same ranges.

The investigations reported in this paper were centred on the influence of the clinical course of calving, and of the mode and timing of calving assistance, on the acid–base balance of the dam and newborn calf.

Materials and methods

A total of 52 dams and their calves were involved in the study. The calvings took place partly in a Holstein-Friesian dairy herd, partly in an animal hospital. All calves were born in anterior presentation.

The acid–base parameters of each dam and calf were determined in blood samples withdrawn from the jugular vein. The system of assays was described earlier (Szenci et al., 1982). The base excess (BE) and extracellular base excess (EBE) were determined with regard to differences in haemoglobin and total serum protein concentration of the dams and their calves as proposed by Szenci and Nyírő (1981).

The dams and their calves were assigned to experimental groups by the mode of assistance required, as follows:

Group 1: Cases without calving assistance (n = 19);
Group 2: Cases requiring assistance by calf-puller (n = 7), for labour failed 30–60 min after the fetal membranes had appeared;
Group 3: Cases requiring Caesarean section (n = 14), which was performed within 4–5 h following rupture of membranes;
Group 4: Cases in which calving assistance was rendered in a late stage, 8–18 h after the beginning of calving (n = 10);
Group 5: Cases in which prepartal disease incidence necessitated Caesarean section to save the calf (n = 2).

The blood samples were withdrawn from the calves immediately after birth and from the dams of groups 1 and 2 within 15 min after expulsion of the fetus, whereas from those of groups 3, 4 and 5 before the beginning of calving assistance.

Results

The results are shown in Tables I, II and III. At birth the calves born without calving assistance (Group 1) had a slight combined respiratory-metabolic acidosis, which is physiological according to the literary data. At the same time, the dams showed a compensated metabolic alkalosis, owing to a slight increase in the HCO_3^- concentration. Moore (1969) observed a slightly

elevated mean pH (7.44 ± 0.06) and a slightly lowered mean pCO_2 (41.0 ± ± 5.9 mm Hg; 0.133 × mm Hg = kPa) in postparturient dams, but both values showed great individual variations (pH = 7.32–7.52; pCO_2 = 31.8–51.0 mm Hg). A similar blood pH (mean: 7.442; range: 7.397–7.470) was found by other authors (Mülling et al., 1972).

The calves of Group 2, delivered by calf-puller, were born in a state of distinct respiratory-metabolic acidosis, whereas the dams of the same group showed a distinct shift of the acid–base balance towards metabolic alkalosis. Moore (1969) obtained similar values for the acid–base parameters (pH = = 7.49–7.47; pCO_2 = 42.5—42.8 mm Hg; HCO_3^- = 31.3–30.6 mmol/l) in two cases of difficult calving.

The acid–base values of the calves delivered by Caesarean section within 4–5 h after the beginning of parturition (Group 3) were scarcely lower than physiological, and those of their dams fell into the physiological range altogether. Amman et al. (1974) found a higher mean pH (7.49 ± 0.03) and HCO_3^- (27.5 ± 2.7 mmol/l) but a lower mean pCO_2 (37.2 ± 3.6 mm Hg) than we did in dams delivered of their calf by Caesarean section.

The dams given late calving assistance (Group 4) were assigned to two subgroups, depending on the physiological or slightly acidotic values of the acid–base parameters. In the first subgroup dystocia was associated with weak or inefficient labour, while in the second subgroup the dams exhausted

Table I

Mean acid–base parameters ($\bar{x}$ ± SD) of the dams and newborn calves

Animal	Parameter	Group 1	Group 2	Group 3	Group 4A	Group 4B
Dam	n	19	7	14	5	5
	pH	7.402±0.054	7.435±0.029	7.395±0.031	7.378±0.026	7.312±0.049
	pCO_2 (kPa)	6.5±0.7	6.3±0.6	5.6±0.4	6.1±0.6	5.1 ±0.5
	BE (mmol/l)	4.6±3.7	6.2±3.6	0.7±2.1	1.5±2.9	—5.9±1.6
	EBE (mmol/l)	4.8±3.8	6.4±3.9	0.5±2.2	1.6±3.1	—6.0±1.5
	HCO_3^- (mmol/l)	29.1±3.6	30.3±3.9	24.7±2.1	26.0±3.1	18.8±1.3
Newborn calf	pH	7.249±0.034	7.185±0.141	7.178±0.099		
	pCO_2 (kPa)	7.9±0.5	8.3±1.7	8.9±0.8	Calves either died *in utero* or were born with a severe metabolic acidosis	
	BE (mmol/l)	—1.9±2.2	—5.0±5.8	—3.9±5.0		
	EBE (mmol/l)	—1.8±2.3	—4.9±5.5	—2.6±4.9		
	HCO_3^- (mmol/l)	24.9±2.2	22.8±3.4	24.1±4.0		

Table II

Blood parameters of acid–base status of a Holstein-Friesian dam (2 years old) and her calf

Parameter	Dam			Newborn calf		
	—1 day	0 day**	+1 day*	Post partum	10 min post partum	1 h post partum*
pH	6.980	7.040	6.920	7.070	6.926	6.950
pCO_2 (kPa)	7.0	6.3	5.0	6.3	6.3	10.0
BE (mmol/l)	—19.1	—17.3	—23.9	—14.9	—20.3	—13.8
EBE (mmol/l)	—17.1	—15.9	—22.2	—14.2	—19.4	—13.2
HCO_3^- (mmol/l)	11.9	12.3	7.3	13.1	9.4	15.7

The fetus was removed by Caesarean section on day 260 of gestation because of ruminitis
* The dam and the calf died after the last blood sampling.
** Before Caesarean section.

by continuous strain had been unable to stand up. In both subgroups, the calves died either *in utero* (n = 7) or were born (n = 3) with a severe respiratory-metabolic acidosis (pH = 6.950–7.002; pCO_2 = 9.5–10.0 kPa; BE = = — 13.5– —14.3 mmol/l; EBE = —11.4– —13.2 mmol/l; HCO_3^- = 15.7–17.1 mmol/l).

The dams which had become clinically ill (ruminitis, hepatic coma) in the prepartal stage (Group 5) showed a shift of the acid–base parameters to the acidotic range. Their calves, delivered by Caesarean section, were born in a state of severe metabolic acidosis (Tables II and III).

Table III

Blood parameters of acid–base status of a Black Pied Lowland x Jersey dam (6 years old) and her calf

Parameter	Dam* before C.s.	Newborn calf**		
		Post partum	1 h post partum	4 h post partum
pH	7.220	7.052	7.076	7.209
pCO_2 (kPa)	4.4	7.2	7.8	8.2
BE (mmol/l)	—12.7	—14.5	—12.3	—4.2
EBE (mmol/l)	—12.8	—13.2	—11.0	—3.0
HCO_3^- (mmol/l)	12.9	14.4	16.4	23.6

The fetus was removed by Caesarean section on day 280 of gestation because of hepatic coma.
* The dam died on the day of Caesarean section (C. s.).
** The calf continued to develop normally further on.

Discussion

Parturition, although a physiological process, is a great stress for both dam and fetus even in uncomplicated cases. The degree of stress varies with age and individual sensitivity, and is precisely portrayed by changes in certain blood parameters. The blood of the parturient dam shows eosinopenia, neutrophilic leucocytosis, lymphopenia, elevated lactate concentration and hypercortisolaemia (Bostedt and Berchtold, 1968; Haraszti et al., 1980; Hunter et al., 1977). Reeves et al. (1972) observed similar alterations in the differential leucocyte count of newborn calves. However, calves born spontaneously, without assistance, may also show an elevated lactate concentration and hypercortisolaemia (Massip, 1980; Mülling et al., 1979). The rise of plasma glucocorticoid may be consequent upon fetal adrenocortical hyperfunction, which is probably involved in the initiation of parturition (Hunter et al., 1977). Although the peripartal metabolic changes have been extensively studied, reports are scanty on simultaneous examinations of maternal and fetal acid–base balance immediately after calving. In the sample survey reported here, the acid–base parameters of dams (Groups 1 and 2) were found in the physiological range (pH, pCO_2), or were very close to it (BE, EBE, HCO_3^-). This observation agrees with that of Mülling et al. (1979), who found a relatively constant pH (7.451 → 7.459) in spite of the elevated lactate concentration (9.13 → 13.17 mg/100 ml) in the blood of the affected dams from 70 min before the end of parturition until the end of it. This finding also indicates that the acid–base parameters of the cows fluctuate within the physiological range if calving is uncomplicated.

Although the Caesarean section spares not only the calf (Eigenmann et al., 1981) but also the dam, to judge from the physiological or practically physiological values of the acid–base parameters after such intervention, it is not advisable to resort to it too frequently. Contemplating calving assistance, one should always bear in mind that, further to economic considerations, the method and timing of assistance should be such as saves the breeding value of the dam and simultaneously prevents excessive shift of the fetal acid–base balance towards metabolic acidosis.

If the dam becomes diseased prepartally (parturient paresis, hepatic coma) and makes a slow recovery, death of the fetus *in utero* can hardly be prevented (Szenci et al., 1982). Since in such cases a shift of the acid–base balance may associate with the basic disorder, it is recommended to test the acid–base parameters and apply aimed therapy ($NaHCO_3$ infusion) if required. Held (1983) observed metabolic acidosis in 7 of 14 preparturient diseased dams. If a disturbance of the acid–base balance is suspected but cannot be substantiated in the field, a Caesarean section should be performed to save the fetus.

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ROLE OF ACID-BASE DISTURBANCES IN PERINATAL MORTALITY OF CALVES

(A SUMMARY OF THESIS)

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Keywords. Acid–base balance, disturbance, newborn, calf, cow, fetus, dam, calving, perinatal mortality.

The profitability of cattle breeding is greatly influenced by the rate of calves born alive and reared to adulthood. In spite of the speedy development of animal breeding, perinatal mortality is still very high (4 to 7%) and constitutes approximately a half of the total calf losses (Anderson and Bellows, 1967; Voelker, 1967; Rodolph, 1970; Walser, 1975). Perinatal mortality (stillbirth) is interpreted as the death of mature calf fetuses during calving or in the first 24 hours of postnatal life. Annual economic losses resulting from stillbirths were estimated at 50 million marks in the Federal Republic of Germany by Walser in 1972, over 200 million dollars in the United States of America by Oxender et al. in 1973, and at 50 million Dutch guldens in the Netherlands by Remmen in 1976. In Hungary, the annual loss may be estimated to be 150 million Forints.

Hahnsdorf (1967) and Greene (1979) failed to detect any pathological change in 73 to 75% of the calves died in the perinatal period. Direct and indirect asphyxia and anoxia were suggested as the causes of death. Since, due to disturbances occurring during parturition in the uteroplacental circulation as a result of the rupture of fetal membranes and uterine contractions, all calf fetuses develop a more or less severe hypoxia and a consequent acidosis, the present investigations were focussed on the determination of the acid–base balance.

In human medicine, Saling (1961) was the first to report on the assessment of the acid–base balance of newborn children. In veterinary practice, the first investigation of this type, involving 10 newborn calves, is associated with the name of Moore (1969). Later on, Mülling et al. (1972) studied the blood pH values of newborn calves, whereas Ammann et al. (1974) the blood pH, pCO_2 and actual hydrogen carbonate (act. HCO_3^-) values of viable and asphyxial calves. A more detailed investigation into the acid–base balance of newborn calves was started in 1977; since that time, research of this type has been initiated in an ever increasing number of countries. At present it is indispensable

for the veterinarian to have knowledge of the physiological events taking place during parturition in the fetus and dam, in order to allow a soonest possible recognition and averting of dangers threatening the fetus.

In the present work, in addition to a statistical survey of the incidence of stillbirths, another aim was to follow up, in the first 48 hours of life, the changes occurring in the acid–base balance of calves born unaided or with assistance (traction or Caesarean section). A further objective of this work was to elaborate a method which would allow an assessment of the newborn calf's general health status (which is closely related to the acid–base status) without laboratory examinations and, accordingly, to take the proper therapeutic measures in due time. The studies included determination of the dam's acid–base balance as well, since, due to the scarcity of pertinent literary data, the degree to which acid–base disturbances of the dam influence the viability of the fetus is poorly known.

The investigations consisted of the following parts:

(1) Survey of the incidence of perinatal calf mortality in large-scale dairy farms;
(2) Measurement and calculation of parameters determining the acid–base balance of newborn calves and their dams;
(3) Follow-up of the changes occurring in the acid–base balance of calves in the perinatal period;
(4) Examination of the relationship between the acid–base balance of the dam and fetus, with particular respect to ruminants;
(5) Status-diagnosis and treatment of newborn calves exhibiting asphyxia.

Results

(1) *Incidence of perinatal mortality*

No Hungarian surveys are available on the incidence of perinatal mortality. To fill this gap, data of 163 dairy farms of 59 state farms, including the calvings of a total of about 140,000 cows for the years 1978 and 1979 (Szenci and B. Kiss, 1982) as well as those obtained in two state farms in a six-year period (Szenci et al., 1981*b*) were processed. The surveys conducted so far were restricted to stocks of less than 100 cows. In the present material the proportion of such stocks was below 5%.

The incidence of stillbirths was examined, broken down and grouped by breeds, years, months, calvings of heifers and cows, sex of fetuses, quantity of milk produced, farm size, number of calving attendants working in the calving premises, training level of attendants in charge of night duty and attendance on bank holidays, construction (type) of the calving premises, calving technology, and dry management practice, separately.

(2) *Measurement and calculation of parameters determining acid–base balance*

The metabolic and respiratory parameters determining acid–base balance relate to human arterial blood of 37 °C temperature. Since from newborn calves arterial and capillary blood samples are difficult to withdraw (Waizenhöfer and Mülling, 1978), blood sampling from the jugular vein has become the generally applied practice. This fact has to be kept in mind when evaluating the acid–base balance, since in the venous blood the pH is lower and the pCO_2 is higher than in the arterial blood (Waizenhöfer and Mülling, 1978). With regard to the fact that the average body temperature of newborn calves is 39 °C, a fact confirmed also by my own examinations (Table I), the temperature-dependent pH and pCO_2 values (Havas, 1980) measured at 37 °C must be corrected for 39 °C. In the calculation of metabolic parameters, i.e. base excess (BE) and extracellular base excess (EBE), it should be taken into consideration that the constants of the van Slyke's equations constructed for human data (equations 1 and 2; Siggaard-Andersen, 1977) depend on the total protein and haemoglobin concentration; therefore, to render possible a more precise calculation, due to the different total protein and haemoglobin concentrations (the haemoglobin and total protein concentration of human blood is 9.43 mmol/l and 70 g/l, respectively; the respective parameters for 2 to 10 weeks old calves are 7.18 mmol/l and 58 g/l; Vagher et al., 1973), the constants depending on the above parameters should be corrected as follows:

$$BE = [(\text{act. } C_{HCO_3^-} - 24.4) + (2.3 \times C_{Hb} + 7.7 \times \times (pH - 7.4)] \times (1 - 0.023 \times C_{Hb}) \quad (1)$$

$$EBE = [(\text{act. } C_{HCO_3^-} - 24.4) + (2.3 \times 3.77 + 7.7) \times \times (pH - 7.4)] \times (1 - 0.023 \times 3.77) \quad (2)$$

Table I

Body temperature of viable calves born unassisted, in anterior presentation, in the first 48 h of postnatal life

Time	Heifer calvings		Cow calvings		Total
n	8	9	10	14	41
pp*	39.6±0.4	39.1±0.7	39.1±0.4	39.2±0.7	39.2±0.5
1 h	39.3±0.4	38.9±0.4	38.9±0.5	39.0±0.3	39.0±0.4
4 h	38.8±0.2	38.9±0.4	38.7±0.2	38.7±0.2	38.7±0.2
24 h	38.5±0.2	38.6±0.2	38.5±0.3	38.6±0.3	38.5±0.2
48 h	38.9±0.6	39.2±0.3	39.2±0.5	39.1±0.3	39.1±0.4

pp*: immediately post partum

In equations (1) and (2), instead of the constant 7.7, a value of 6.38, and in equation (2) instead of $C_{Hb} = 3.77$ a value of $C_{Hb} = 2.87$ should be used (Szenci and Nyírő, 1981).

With regard to the above facts, the BE and EBE values of dams and calf fetuses cannot be compared, as the haemoglobin and total protein concentrations of cows (6.8 mmol/l and 77 g/l, respectively) differ from those of calf fetuses (5.3 mmol/l and 44 g/l, respectively; Shalm et al., 1975). Thus, in the case of dams the constant (7.7) and C_{Hb} value of equations (1) and (2) should be corrected for 8.47 and 2.7, respectively, while in the case of fetuses for 4.84 and 2.1.

(3) *Alterations in the acid–base balance of calves in the perinatal period*

3.1 *Acid–base balance of calves born unaided and with assistance, immediately post partum*

In agreement with data obtained for humans, the average blood pH and BE values of calves born unassisted in anterior presentation do not decrease below 7.2 and −3 mmol/l, respectively; therefore, such a mild, combined respiratory-metabolic acidosis can be regarded as physiological (Table II). The differences found between the acid–base parameters of heifer- and bull--calves born unassisted from primi- and pluriparous dams (Table VI) and twin-calves and calves born spontaneously in anterior presentation unanimously indicate that the shift in the acid–base balance is directly proportional to the body mass of calves and inversely proportional to the measurements of the birth canal. Significant differences existing in pH and metabolic parameters between calves easily extracted by traction (by the use of maximum 3 to 4 men's pulling power) and those less easily extracted because of hip lock flexion (Table III), and the differences found in acid–base status between calves extracted in anterior presentation in different ways (Table IV) unanimously indicate (with the exception of placental and umbilical-cord disorders) that the duration of passage through the birth canal has a decisive importance. This is shown not only by the significant differences found between the duration of traction and the blood pH values of newborn calves (Table V), but also by the fact that there exist no significant differences in acid–base status between calves delivered by primi- and pluriparous dams by Caesarean section (Table IV).

With the exception of placental and umbilical-cord disorders, a severe acidosis (blood pH < 7.0) should be reckoned with (Table V) in the case of a traction lasting about 3 min (in anterior presentation).

The calves extracted with assistance in anterior or posterior presentation were born with an expressed respiratory and metabolic acidosis (blood pH: between 7.2 and 7.0; BE: −3 to −10 mmol/l; Table II).

Table II

Acid–base balance of calves born unassisted or with assistance, immediately post partum ($\bar{x} \pm SD$)

Parameter	Unassisted		With assistance							
	Anterior presentation	Twinning	Anterior presentation			Posterior presentation			F	Values of t
			Traction	Calf-puller	Caesarean section	Traction	Calf-puller	Caesarean s.		
n	41	7	46	15	43	6	8	8		
pH	7.219	7.239	7.136	7.141	7.165	7.126	7.096	7.148	3.3***	0.065*
	0.052	0.064	0.098	0.113	0.137	0.114	0.134	0.124		0.086**
										0.111***
pCO_2 kPa	8.1	8.6	9.7	9.0	8.9	9.9	8.9	9.3	5.6***	0.8
	0.6	1.4	1.4	1.9	1.2	1.5	1.4	1.4		1.0
										1.3
BE mmol/l	—2.9	—0.8	—4.7	—6.3	—4.1	—5.4	—8.4	—4.7	2.4*	3.0
	3.2	1.1	4.0	4.2	6.4	4.5	6.2	6.1		3.9
										5.0
EBE mmol/l	—1.8	—0.6	—3.1	—5.3	—2.7	—3.7	—7.3	—3.3	2.4*	2.8
	3.1	1.1	3.9	3.8	5.9	3.7	5.4	5.9		3.7
										4.7
act. HCO_3^- mmol/l	24.2	26.4	23.8	22.1	23.9	23.5	20.9	23.6	1.8	ns
	2.7	0.7	3.1	2.7	4.7	2.5	4.3	4.9		

*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, ns: not significant

Table III

Acid–base balance of newborn calves easily extracted in anterior presentation and of those less easily extracted because of hip lock flexion, immediately post partum ($\bar{x} \pm SD$)

Parameter	Anterior presentation	
	Easily extracted by traction	Hip lock flexion
n	27	16
pH	7.172 0.081	7.077*** 0.069
pCO_2 kPa	9.8 1.5	9.7 1.2
BE mmol/l	—2.6 2.5	—8.6*** 3.1
EBE mmol/l	—0.7 2.3	—7.5*** 3.3
act. HCO_3^- mmol/l	25.9 1.5	20.7*** 2.6

* $P < 0.001$

Table IV

Acid–base balance of calves born in anterior presentation unassisted and with assistance, immediately post partum ($\bar{x} \pm SD$)

Parameter	Heifer calvings			Cow calvings		
	Unassisted	Traction	Caesarean s.	Unassisted	Traction	Caesarean s.
n	17 (a)	18 (b)	23 (c)	24 (A)	28 (B)	14 (C)
pH	7.196 0.058	7.098*, a 0.103	7.162 0.148	7.234 0.042	7.162**, A 0.089	7.172*, A 0.118
pCO_2 kPa	8.2 0.7	10.2***, a **, c 1.6	8.9 1.3	8.1 0.4	9.4***, A 1.3	9.1**, A 1.2
BE mmol/l	—4.2 3.4	—6.3 4.5	—4.3 6.9	—2.0 2.7	—3.8 4.0	—3.9 5.6
EBE mmol/l	—3.2 3.3	—4.4 4.2	—2.9 6.4	—0.9 2.7	—2.3 3.6	—2.3 5.1
act. HCO_3^- mmol/l	23.1 2.9	23.0 3.5	23.7 5.1	25.0 2.4	24.4 2.9	24.4 4.1

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$
a ⇔ A* = pH, BE, EBE, act. HCO_3^-
b ⇔ B* = pH

Table V

Relationship between the duration of traction and blood pH value of the newborn calf ($\bar{x} \pm SD$)

Group	n	Blood pH	Duration of traction (sec)	Number of helps at traction
Group 1 (pH > 7.2)	24	7.25***, 2, 3 ±0.03	46***, 3 ±32	2.3**, 3 ±1.2
Group 2 (pH 7.2—7.0)	13	7.12***, 3 ±0.05	97***, 3 ±38	3.0 ±1.0
Group 3 (pH < 7.0)	5	6.89 ±0.05	221 ±127	3.6 ±0.5

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

Depending on the mode of assistance, these values decreased, for both presentations, in the following order: Caesarean section, traction and use of calf-puller. In posterior presentation this decrease was more expressed than in anterior presentation (Szenci et al., 1981*a*; Szenci, 1983; Szenci et al., 1984*b*).

3.2 *Acid–base balance of newborn calves delivered by Caesarean section in the period before and after delivery (0 to 60 min)*

In the case of Caesarean sections performed within 4 to 5 h after the rupture of fetal membranes, the acid–base parameters of 67.7% of the calves were within the physiological range. At the same time, 14.7% and 17.6% of the calves showed an expressed and a severe acidosis, respectively. With the exception of umbilical-cord and placental disorders, the degree of intrauterine acidosis depends mainly on the time elapsed until Caesarean section and on the mode of assistance rendered before Caesarean section.

In agreement with data of the literature, up to the 10th min post partum a further decrease of metabolic parameters was observed. However, this decrease was more expressed between blood samples taken before (a. umbilicalis) and after delivery (v. jugularis) than in the first 10 min of postnatal life. From the 10th min, a compensation for the acidosis was started by the buffer systems of the blood and by hyperventilation. In viable calves the degree of compensation reached the significance level, whereas in calves exhibiting pronounced or severe asphyxia it developed at a slower rate (Szenci et al., 1985).

3.3 Changes in the acid–base balance of calves born unassisted or extracted by traction, in the first 48 h of postnatal life

The mild metabolic acidosis of heifer- and bull-calves born spontaneously to primi- and pluriparous dams was compensated already 1 h after birth,

Table VI

Changes in the acid–base balance of calves born unassisted, in the first 48 h of postnatal life ($\bar{x} \pm SD$)

Parameter		Group	pp	1 h	4 h	24 h	48 h
pH	1	♀	7.208±0.049	7.282±0.057	7.309±0.034	7.345±0.024	7.367±0.029
		♂	7.187±0.067	7.241±0.046	7.337±0.038	7.403±0.044***a	7.372±0.015
	2	♀	7.250±0.040	7.303±0.063	7.339±0.042	7.402±0.020***a	7.365±0.021
		♂	7.224±0.042	7.284±0.040	7.307±0.096	7.381±0.045**a	7.379±0.040
pCO_2 kPa	1	♀	8.1±0.9	7.7±0.5	7.1±0.1	6.9±0.8	7.0±0.3
		♂	8.3±0.7	7.9±0.9	7.2±0.4	6.2±0.5	6.3±0.4***a
	2	♀	8.1 ±0.4	7.6±0.7	7.1±0.5	7.1±0.7	6.9±0.1**bd
		♂	8.2±0.5	7.6±0.6	7.0±0.5	6.7±0.9	6.3±0.6***a
BE mmol/l	1	♀	—3.7±2.5	0.3±4.2	0.2±2.5	2.1±1.9**c	3.8±2.3
		♂	—4.8±4.2	—1.7±5.3	2.3±1.9	4.1±4.4*c	1.7±1.8
	2	♀	—1.0±2.7	0.9±2.6	2.1±2.0	7.2±3.1	3.3±1.4
		♂	—2.8±2.6	—0.1±3.2	—0.3±3.9	2.7±3.6**c	2.5±3.2
EBE mmol/l	1	♀	—2.7±2.4	1.0±4.1	0.8±2.5	2.4±2.1**c	4.3±2.2
		♂	—3.7±4.1	—0.9±5.5	2.8±1.6	4.3±4.6*c	1.8±1.8
	2	♀	—0.1±2.8	1.6±2.3	2.7±1.9	7.7±3.3	3.7±1.4
		♂	—1.6±2.6	0.7±3.3	0.1±3.8	2.9±3.7**c	2.6±3.3
act. HCO_3^- mmol/l	1	♀	23.5±2.1	26.6±3.7	26.0±2.3	27.3±2.3**c	29.3±2.1
		♂	22.8±3.6	25.0±5.3	27.8±1.4	28.6±4.4*c	26.4±1.9
	2	♀	25.9±2.5	27.0±1.8	27.7±1.7	32.2±3.5	28.4±1.2
		♂	24.5±2.3	26.3±3.1	25.1±3.3	27.5±3.7**c	27.1±3.3

1) Heifer calving, ♀: n = 8[a] ♂: n = 9[b] 2) Cow calving, ♀: n = 10[c] ♂: n = 14[d] * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$

Table VII

Changes in the acid–base balance of calves born unassisted and with assistance, in the first 48 h of postnatal life ($\bar{x} \pm SD$)

Parameter	Group	pp	1 h	4 h	24 h	48 h
pH	1	7.219±0.052	7.278±0.053	7.321±0.064	7.383±0.041	7.371±0.029
	2	7.239±0.064	7.257±0.088	7.297±0.091	7.351±0.088	7.396±0.039
	3	7.141±0.113	7.254±0.080	7.346±0.046	7.360±0.070	7.386±0.035
	4	7.096±0.134	7.147±0.129	7.273±0.063	7.346±0.046	7.398±0.024
pCO_2 kPa	1	8.1±0.6	7.6±0.6	7.0±0.4	6.7±0.7	6.5±0.5
	2	8.6±1.4	8.3±1.9	7.5±1.4	7.6±1.8	7.2±1.5
	3	9.0±1.9	7.8±1.1	7.6±0.8	7.4±1.0	7.3±0.8
	4	8.9±1.4	7.9±0.8	7.5±0.6	7.0±0.5	5.9±0.2
BE mmol/l	1	—2.9—3.2	—0.1±3.7	0.9±3.0	3.9±3.8	2.7±2.5
	2	—0.8±1.1	—0.4±3.2	—0.1±2.8	3.8±2.1	6.6±2.1
	3	—6.3±4.2	—1.5±4.2	4.3±2.8	5.2±3.6	6.6±2.7
	4	—8.4±6.2	—7.7±5.5	—1.8±2.0	2.2±2.4	2.1±0.9
EBE mmol/l	1	—1.8±3.1	0.6±3.8	1.4±2.9	4.2±4.0	3.0±2.5
	2	—0.6±1.1	—0.2±3.4	0.4±3.1	4.1±1.9	6.9±1.8
	3	—5.3±3.8	—1.1±4.0	4.8±2.8	5.5±3.4	6.8±2.3
	4	—7.3±5.4	—7.1±5.5	—1.3±1.9	2.5±2.3	2.1±0.7
act. HCO_3^- mmol/l	1	24.2±2.7	26.2±3.5	26.5±2.6	28.8±3.9	27.6±2.5
	2	26.2±0.7	26.1±3.2	25.8±1.8	29.4±4.3	31.8±4.7
	3	22.1±2.7	24.9±3.6	30.1±2.8	30.7±4.9	31.7±3.0
	4	20.9±4.3	20.1±3.8	24.3±1.3	27.4±2.0	26.3±0.5

[1] Unassisted (anterior presentation); n = 41
[2] Twin calving; n = 7
[3] Calf-puller (anterior presentation); n = 15
[4] Calf-puller (posterior presentation); n = 8 (1st h: n = 7; 48th h: n = 6)

Table VIII

Significant differences in acid–base balance between calves born unassisted and those delivered with assistance

Blood sampling Parameter	pp		1 h		4 h		24 h		48 h	
	F	*t* values	F	*t* values	F	*t* values	F	*t* values	F	*t* values
pH	**	0.060*	***	0.055	ns		ns		ns	
		0.080**		0.073						
		0.104***		0.094						
pCO_2	*	0.9	ns		*	0.5	*	0.7	***	0.5
		1.2				0.7		0.9		0.7
		1.5				0.9		1.2		0.9
BE	***	2.8	***	3.0	***	2.2	ns		***	1.9
		3.7		4.0		2.9				2.5
		4.8		5.2		3.8				3.2
EBE	***	2.6	***	3.0	***	2.1	ns		***	1.8
		3.4		4.0		2.8				2.4
		4.4		5.2		3.7				3.1
act. HCO_3^-	***	2.1	***	2.7	***	1.9	ns		***	2.2
		2.8		3.6		2.5				2.9
		3.6		4.6		3.3				3.7

* $P < 0.05$; ** $P < 0.01$; ***$P < 0.001$; ns = not significant

whereas their respiratory acidosis was not fully compensated even 48 h later. Compensation was not uniform in the various groups; the metabolic parameters showed statistically significant differences 24 h, while the respiratory parameters even 48 h, after birth (Table VI).

The compensation of the metabolic acidosis of calves born with assistance was protracted depending on the severity of the acidosis, while no difference occurred in the compensation of the respiratory parameter (pCO_2) (Tables VII and VIII; Szenci et al., 1981*a*, 1982*b*).

3.4 *Effect of calving assistance rendered at different times and in different ways on the acid–base balance of dam and fetus*

The acid–base balance of calves to be born is determined primarily by the time elapsed until calving assistance and by the mode of assistance, whereas the acid–base status of dams is characterized by high stability, irrespective of the mode of intervention (except for the use of a calf-puller). The acid–base balance of dams undergoes a shift towards acidosis only in those cases of delayed aid when complicated calving is associated with intense labour, and the continuous strain leads to recumbency of the dam.

In pathological conditions occurring in the immediate prepartal period (ruminitis, coma hepaticum) a shift of the dam's acid–base balance towards metabolic acidosis should also be reckoned with (Szenci, 1985).

(4) *The relationship between the acid–base balance of dam and fetus, with particular respect to ruminants*

4.1 *Effect of experimentally-induced acidosis of the dam on the acid–base status of the fetal calf*

Metabolic acidosis was induced in 7 pregnant heifers and 2 cows in the prepartal period by supplementing the feed with sucrose. Two pregnant heifers served as control.

The objective of these experiments was to study the degree to which experimentally-induced acidosis of the dam can influence the acid–base status and the viability of the fetus and newborn calf.

Based upon the blood and urinary parameters indicative of acid–base status, the dam's acute acidosis lasting one day exerted no adverse influence on the acid–base balance of fetal calves to be born (the acid–base balance of these fetuses was the same as the control values). On the other hand, when the dam showed a progressively aggravating, and finally very severe, acidosis lasting 4 to 7 days, the calves either were born in an acidotic state or developed acidosis within a few hours after birth. Both calves born in such a condition died shortly after birth (Szenci et al., 1982*a*).

4.2 *Effect of experimentally-induced acidosis of pregnant ewes on the acid–base balance and carbohydrate metabolism of fetal lambs*

Since in the previous experiment the possibility could not be fully excluded that neonatal acidosis developed not directly due to the dam's acidosis but as a result of anaerobic glycolysis consequent to an insufficient oxygen supply of the fetus, the subsequent experiments included prolonged administration of NH_4Cl. We hoped that the resulting prolonged acidosis would not be accompanied by disorders in fetal oxygen supply. In the blood of ewes treated with ammonium chloride in the last stage of pregnancy (0.1 to 0.5 g NH_4Cl/kg body mass/day per os, continuously) a non-compensated metabolic acidosis developed; as opposed to this, the acid–base balance of fetal lambs suffered only very slight changes. More considerable alterations were found for the acid–base balance of the fetal fluids, as indicated by the mean pH values of both the allantoic and the amniotic fluids which were significantly lower than those found for the control group (Kutas and Szenci, 1983). As regards carbohydrate metabolism, no statistically significant difference was found by comparing the mean blood glucose concentrations of the two groups. However, it was striking that in the liver of NH_4Cl-treated ewes the glycogen stores were almost completely depleted (with the exception of the centrolobular part of the hepatic lobules); at the same time, fetal liver cells contained normal amounts of glycogen. Since, despite the maternal acidosis, the activity of enzymes of placental origin (lactate dehydrogenase and carbonic anhydrase) failed to

undergo considerable changes, it can be postulated that the fetoplacental gas exchange was normal during the experiment (Szenci et al., 1984*a*). The above results allow us to conclude that, owing to the selective functioning of the epitheliochorial placenta and the increased acid excretion of the fetal kidneys, the fetus is protected against acid–base disturbances of maternal origin provided that the fetomaternal gas exchange remains undisturbed. Since a prolonged metabolic acidosis is accompanied by an impairment of cardiac function and blood circulation as well as by a fall of blood pressure, a disturbance in uteroplacental gas exchange, i.e. an intrauterine asphyxia, should be reckoned with.

(5) *Status-diagnosis and treatment of newborn calves exhibiting asphyxia*

5.1 *Status-diagnosis of newborn calves exhibiting asphyxia*

The applicability of the status-diagnosis score system elaborated on the basis of practical experiences gained in the field of human medicine has been proved by the statistically significant correlation existing between the tonicity of the newborn calf immediately post partum and the parameters characterizing the acid–base balance (Szenci, 1982*).

This score system enables us to assess the newborn calf's general status, which is in association with the acid–base balance, without laboratory measurements, and permits to take the necessary therapeutic measures in due time. As compared with the modified Apgar scheme and with the blood-test-based diagnosis of slight and severe asphyxia, an important advantage of the present method is that even persons having only a basic special training can learn it. According to recent experience, mainly in determining the vitality of Caesarean-derived calves immediately post partum, an erroneous assessment may stem from the fact that as much as 2 to 3 min may elapse from the time when the calf lifts up its head until it changes its position from lateral to oblique abdominal recumbency. In such cases errors can be excluded by examining the calf's response to throwing cold water over its head.

5.2 *Treatment of newborn calves exhibiting asphyxia*

After having cleared their respiratory passages, calves born with reduced vitality (V-II) require no therapy except physical stimulation and stimulation of the respiratory centre with Dopram®–V (Szenci et al., 1980) or Respirot® (Köchli, 1969).

* V-O: toneless, head drooping, limbs extended, cardiac activity absent
V-I: toneless, head drooping, limbs extended, cardiac activity present
V-II: low tonicity, abdominal recumbency with head requiring support; reduced number and intensity of reflectoric movements
V-III: normal tonicity, head erect, normal reflectoric movements (Szenci, 1982)

Table IX

Results of therapy of newborn calves exhibiting asphyxia, in the first 48 h of postnatal life

Treatment Vitality (degree)	VF-II		VF-I	
	lives	died* (%)	lives	died* (%)
Physical stimulation	24	1 (4.2)	6	3 (50.0)
Dopram® — V	22	1 (4.5)	11	5 (45.5)
Dopram® — V + $NaHCO_3$–glucose infusion	—	—	11	4 (36.4)
Total	46	2 (4.3)	28	12 (42.9)

* In the first 48 h of postnatal life

Respiratory stimulants must be given only in cases when, despite physical stimulation, the onset and normal rhythmicity of respiration is delayed.

In addition to the above treatment, it is expedient to give calves showing severe asphyxia (V-I) buffer therapy ($NaHCO_3$ and glucose infusion) as well (Müller, 1974; Walser and Maurer-Schweizer, 1978), since in the absence of such a therapy the compensation of severe respiratory and metabolic acidosis will take a longer time, the newborn calves will stand up later, and the uptake of sufficient quantities of colostrum will be delayed (Szenci, 1982). All these factors predispose the neonatal calf to *E. coli* enterotoxaemia (Eigenmann et al., 1982).

The recovery chances of newborn calves are reduced by the per-diapedesim haemorrhages of hypoxic origin (in the brain and spinal cord; Haughey, 1975), the fetal fluids got into the lungs (Eigenmann et al., 1982) as well as the pronounced oedematous infiltration of the tongue and buccal region despite the complex therapy applied. The results obtained by the present author so far (Table IX) indicate that when severe acidosis can be traced back to a stress of relatively short period (to the duration of passage through the birth canal in the case of traction), rapid improvement can be achieved by physical stimulation and administration of drugs stimulating the respiratory centre. This is in agreement with the observations of Eigenmann et al. (1982). In all cases when acidosis has been existing for a long period (delayed assistance, prolonged traction), the efficacy of therapy is poorer than expected, despite the $NaHCO_3$–glucose infusion (Eigenmann et al., 1982), since the prolonged presence of acidosis allowed the above-listed changes and lesions to develop. The duration of acidosis has great prognostic importance in human medical practice as well (Kerpel-Fronius, 1972).

Recommendations for the practical application of the results

At present, in veterinary practice the main emphasis should be laid on the prevention of asphyxia of calves to be born, since, on the one hand, instru-

ments suitable for a reliable clearing of respiratory passages and for the maintenance of this state, and for artificial respiration of calves under practical conditions are not yet available; and, on the other hand, profitability factors still play a decisive role. The most important breeding objectives can be achieved only by creating the necessary *managemental* (regular movement of animals in the dry period), *feeding* (avoidance of obesity) and *organizational* (adequate rearing and breeding of heifers, an adequate training level of calving attendants, ensuring a continuous attendance to cows calving at night or on bank holidays) *conditions* (to mention only the most important ones), which may lead to a reduction in the number of calving assistances required. This is even more important since calving assistance in itself may result in a shift of the calf's acid–base balance. In large-scale farms, appointment of the actual tasks is largely facilitated by a continuous registration, and monthly evaluation, of the course of calvings.

In the case of difficult calvings, the mode and time of calving assistance should be chosen with regard to profitability factors and in a manner to allow the least possible shift of the calf's acid–base balance towards acidosis. Before applying traction, the measurements of the soft birth canal should always be considered. When dilatation of the soft maternal passages is not sufficient, they must be expanded nonsurgically or surgically (episiotomia lateralis) and forewaters should be supplemented to avoid tractions longer than 2 to 3 min. In the case of hip lock flexion, the respiratory passages must be cleared to avoid severe asphyxia. Throwing cold water over the calf's nape and administration of respiratory stimulants are also expedient. Traction should be discontinued until respiration has become rhythmical. In posterior presentation the above measures are especially important, since untimely strangulation of the umbilical cord in itself endangers the life of the fetus.

If a prolonged traction would be expected (based upon the size of the fetus and the measurements of the maternal passages as related to one another or, in doubtful cases, from the result of the short test traction), Caesarean section should be carried out to save the calf and to prevent injuries of the maternal passages. In the case of Caesarean sections, the sooner the surgical intervention takes place after the rupture of fetal membranes, the lower the proportion of newborn calves with severe asphyxia will be. The most recent studies have shown that before making a decision as to the mode of calving assistance in an animal hospital, the results of acid–base balance determination from blood samples withdrawn from the v. metacarpalis superficialis volaris or v. metatarsalis dorsalis lateralis (Waizenhöfer and Brattig, 1975; Szenci and Bakonyi, 1985) or from the v. digitalis dorsalis communis III dorsomedialis (Eigenmann, 1981) should be considered.

The routine use of complex treatment ($NaHCO_3$ and glucose infusion, stimulation of circulation, prophylactic use of antibiotics) of calves born

with severe asphyxia (V-I) reduces postnatal calf losses. In addition to an adequate therapy (buffer therapy based upon the determination of acid–base balance), in the case of calves with asphyxia particular attention should be paid to the ingestion of sufficient amounts of colostrum, since the lack of colostrum uptake is accompanied by an increased susceptibility to infections *(E. coli)*.

The results obtained in studies on the experimentally-induced metabolic acidosis of dams have theoretical significance in the first place. However, it is remarkable that, due to the selective functioning of the placenta on the one hand and to the acid-excreting activity of the fetal kidneys on the other, fetuses of dams having an epitheliochorial placenta are protected until the cardiac and circulatory insufficiency consequent upon severe metabolic acidosis upsets this protective mechanism.

All these results call the attention to the necessity of establishing veterinary neonatology as an independent branch of science. Only this could bring about a change of the attitude according to which in cases of difficult calvings the dam should be saved in the first place; a view still held at most clinics of obstetrics.

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ELECTROCARDIOGRAPHIC SIGNS IN VENTRICULAR REPOLARIZATION OF EXPERIMENTALLY INDUCED HYPOKALAEMIA AND APPEARANCE OF THE U-WAVE IN DOGS

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Hypokalaemia was induced experimentally in 11 dogs. The S–T segment, the shape of the T-wave, and the appearance of the U-wave were studied by electrocardiograms taken daily. In addition to the three standard leads (I, II and III) and to the unipolar limb leads (aVR, aVL and aVF), three chest leads (CV_6LU, CV_6LL and CV_5RL) and a dorsal (V_{10}) lead were used.

Based upon the five-day study it was established that the number of pathological ST deviations rose proportionately to the significant decrease of the serum potassium level both in the experimental animals and in the various leads. Of the leads used, deviations were most frequent in leads II, aVF, and III.

In the standard and unipolar limb leads the T-wave gradually became lower, and the F value determined by analysis of variance showed a high level of significance. As compared to the mean values obtained at baseline examination, on the 5th day a significance of $P < 0.001$ was obtained in all of the leads. The T-waves tended to become lower also in the chest leads and in the dorsal lead; however, in these leads the differences were not significant.

Besides the signs indicative of disturbances in ventricular repolarization, the U-waves, whose appearance was in direct relation to the drop of the serum potassium level, are considered characteristic of hypokalaemia.

Keywords. Hypokalaemia, electrocardiography, S–T segment, T-wave, U-wave, dog.

In dogs, disturbances of the electrolyte and water metabolism are frequently encountered. As a rule, these disorders do not occur independently, but join primary diseases characterized by vehement vomiting, diarrhoea and polyuria. Of the electrolyte disturbances, hypokalaemia consequent upon potassium loss has a particular significance since it influences the functioning of both the striated and the smooth muscles. In addition to leading to a metabolic disturbance of extracellular origin, the decrease of serum potassium level causes a considerable myocardial dysfunction as well. Alteration of the electrolyte gradient decreases the contractility of muscle fibres, leads to disturbances in impulse formation and conduction, and initiates necrobiotic processes (Antalóczy, 1976).

In hypokalaemia, the membrane structure and arrangement of myocardial fibres and the mode and time of ion transport differ from those found under physiological conditions, resulting in a disturbed repolarization.

Ventricular repolarization is represented by the S–T segment and the T-wave. The S–T segment is the "slow" part of repolarization, which takes its origin at point J (junctional point) of the ventricular complex and terminates

with the beginning of the T-wave. The "rapid" part of repolarization is demonstrated by the T-wave, whose terminal point returns to the isoelectric line.

Large deviations in the S–T segment and alterations of the T-wave are indicative of ischaemia, necrosis or metabolic disturbances of the ventricular muscles. In animals, these changes are seen most frequently in general anoxia (following anaesthesia or asphyxia), in insufficient blood supply of the coronary arteries (e. g. in the case of insufficient ventricular pressure), in infections or inflammations (endocarditis, epicarditis), and in disturbances of the electrolyte balance, particularly in cases of renal insufficiency and endocrine disorders (Darke, 1974; Rubin, 1968).

According to Bertram (1980), changes in the S–T segment and the T-wave are always indicative of myocardial damage. If the electrode is placed over a myocardial area damaged because of ischaemia, an elevation, while with the electrode placed far from the damaged area a depression, of the S–T segment will be observed. In narcosis, a gradual increase in the amplitude of the T-wave is indicative of myocardial hypoxia, and sudden deviations of the S–T segment in either direction or an inversion of the T-wave indicate myocardial ischaemia.

Studying the standard leads of 50 healthy, anaesthetized dogs, Lombard and Witham (1955) observed S–T segment deviations of 0.09 mV in 30%, and those of 0.10 to 0.20 mV in 48% of the animals. In the chest leads the potential of deviation was between 0.10 and 0.19 mV in 40%, and higher than 0.20 mV in 18% of the dogs. The T-wave was more varied, since its amplitude was between −0.30 and 0.15, −0.45 and 1.00, and −0.25 and 0.70 mV in leads I, II, and III, respectively.

According to Nemec (1971), in standard leads depressions exceeding 0.20 mV and elevations higher than 0.15 mV of the T-wave, as well as a deep negative T-wave, can be considered pathological, particularly if accompanied by other signs.

Ettinger and Suter (1970) considered the S–T segment pathological if in leads II and III its depression and elevation exceeded 0.20 mV and 0.15 mV, respectively; and, if in the chest lead CV_6LL its depression was larger than 0.30 mV, and in the leads CV_6LL and CV_6LU its elevation was higher than 0.30 mV.

In spite of its great variability, the T-wave is always regarded as pathological if its amplitude exceeds 25% of that of the R-wave (Ettinger and Suter, 1970; Clerc, 1975).

In our opinion, in the standard leads S–T segment elevations exceeding 0.15 mV and depressions > 0.20 mV, while in the chest leads deviations exceeding 0.30 mV can be considered pathological.

Under physiological conditions, no U-wave can be detected in the ECG of dogs; the appearance of a U-wave is always regarded as pathological. In hu-

mans, U-wave appears in part of the cases also in normal circumstances; however, its significance has not been fully elucidated yet. According to Unghváry (1963), it develops through the difference between negative post-potentials of action currents. In man, the U-wave is considered pathological if it is negative, diphasic, and shows a discordant course as compared to the T-wave.

Considering the practical significance of electrolyte and, particularly, potassium metabolism, we studied the changes occurring in the ventricular repolarization portion of the canine ECG during experimentally induced hypokalaemia lasting several days.

Materials and methods

Eleven clinically healthy mongrel dogs of either sex, aged 2 to 4 years, with a body mass between 8 and 17 kg were used. After the baseline values had been recorded (day 1), the dogs were given an aqueous solution of a cation-exchanger containing 1 g/kg body mass Na-polystyrolsulphonate (Resonium A®, Winthrop GmbH), twice daily, for 5 days, per os, through an oesophageal tube. In addition, to achieve the desired hypokalaemia, hydrochlorothiazidum tablets (Hypothiazid®, Chinoin, Budapest) were given in a dose of 10 mg/kg body mass, per os. The dogs were kept in individual cages throughout the experiment, and received dog food (KUKE delicacy) and drinking water ad libitum.

Besides the regular clinical observation, blood samples were taken daily from the jugular vein to determine the K^+ and haematocrit values.

The K^+ concentration was determined in serum and in the whole blood by flame photometry (Pulfrich), and the haematocrit value with an Erythrovolmet instrument (Fok-Gyem, Budapest). Intracellular K^+ was calculated from these data.

Electrocardiograms (ECG) were made of the dogs daily with an ER-31 type (Medicor, Budapest) three-channel, direct-recording electrocardiograph. ECGs were taken of all the dogs in right lateral recumbency using, in addition to the three standard leads of Einthoven (I, II, III) and the three unipolar limb leads of Goldberg (aVR, aVL, aVF), three chest leads (CV_6LU, CV_6LL, CV_5RL) and a dorsal (V_{10}) lead.

From the obtained results daily means were calculated and compared by a one-way analysis of variance.

Results

At the beginning of the experiment, the mean serum potassium level of the 11 dogs was 4.31 mmol/l; upon the effect of the administered drugs and parallel with time, this level decreased to 3.07 mmol/l by day 5. The F value

of the analysis of variance was highly significant; as compared to the baseline value, on day 2 the significance level was $P < 0.01$ while on the subsequent days $P < 0.001$.

The K value of whole blood underwent a slight decrease, whereas intracellular K^+ remained constant. The slight increase observed for the haematocrit value was within the physiological range (Table I).

At the beginning of the experiment, higher-than-physiological deviations of the S–T segment from the isoelectric line were found in a total of 12 leads of 4 dogs. The number of pathological deviations increased proportionately to the decrease of serum potassium level until day 4 both in the experimental

Table Ia

Mean values of potassium ion concentration and haematocrit during the 5-day period of study

Days	Potassium, mmol/l			Haematocrit
	in serum	in whole blood	intracellularly	
Day 1	4.31	6.21	8.23	0.49
Day 2	3.72	6.07	8.28	0.52
Day 3	3.44	5.98	8.26	0.53
Day 4	3.19	5.76	8.29	0.52
Day 5	3.07	5.69	8.17	0.53
$P < 0.05$	0.40	0.68	1.21	0.07
$P < 0.01$	0.52	0.88	1.62	0.09
$P < 0.001$	0.69	1.17	2.11	0.13

Table Ib

Analysis of variance of mean K^+ concentration and haematocrit values

Parameter	SQ	FG	MQ	F
Potassium–serum				
Total	21.46	54		
Treat (between days)	10.68	4	2.67	12.38***
Error	10.78	50	0.22	
Potassium–whole blood				
Total	33.39	54		
Treat (between days)	2.00	4	0.50	0.80
Error	31.39	50	0.63	
Potassium–intracellular				
Total	101.29	54		
Treat (between days)	0.11	4	0.02	0.01
Error	101.18	50	2.02	
Haematocrit				
Total	3786.54	54		
Treat (between days)	185.63	4	46.40	0.64
Error	3600.91	50	72.02	

SQ = sum of squares; FG = degrees of freedom; MQ = mean squares; F = F ratio

Table II

Incidence of S–T segment alterations* (mV) in the various leads during the 5–day period of study

Lead Day	I	II	III	aVR	aVL	aVF	CV_6LU	CV_6LL	CV_5RL	V10	Total	Incidence in dogs
1		4	2		1	2	1	1	1		12	4
2	1	5	5	3	4	5	4	2	2	1	32	9
3	1	6	4	2	2	4	2	2	6	3	32	9
4	3	8	6	5	1	7	3	1	1		35	10
5		7	4	1	2	6	3	2	2		27	9
Total	5	30	21	11	10	24	13	8	12	4	138	

* Standard leads: elevation > 0.15 mV, depression > 0.20 mV; praecardial and dorsal leads: elevation > 0.30 mV, depression > 0.30 mV

animals and in the various leads. On day 5 a slight decrease was noted as compared to values obtained on the day before. During the 5-day period, the total number of S–F segment deviations from physiological was 56, 45 and 33 in the standard, unipolar limb, and chest leads, respectively. Deviations were most frequent in leads II, aVF and III (Table II).

During the 5-day study, the T-wave gradually became lower in the standard and unipolar limb leads. The F value of lead I, determined by mean-value analysis of variance, showed a significance of medium degree, while those of the other two standard leads and the three unipolar limb leads were highly significant. As compared to the mean baseline values, on day 5 a significance of $P < 0.001$ was obtained in all the applied leads.

T-waves tended to become lower also in the chest and dorsal leads; however, these differences were not significant (Tables III and IV).

At the baseline examination, U-wave was not observed in any of the leads used. Parallel to the decrease of the mean potassium concentration of the

Table III

Alterations in mean values of the T-wave (mV) in the various leads during the 5-day period of study

Lead Day	I	II	III	aVR	aVL	aVF	CV_6LU	CV_6LL	CV_5RL	V_{10}
1	0.101	0.259	0.210	0.171	0.128	0.228	0.449	0.392	0.437	0.174
2	0.079	0.173	0.132	0.134	0.087	0.159	0.348	0.302	0.375	0.193
3	0.068	0.144	0.134	0.100	0.081	0.135	0.326	0.263	0.336	0.173
4	0.060	0.126	0.114	0.084	0.055	0.125	0.320	0.269	0.301	0.152
5	0.036	0.102	0.089	0.065	0.042	0.103	0.268	0.215	0.259	0.142
$P < 0.05$	0.042	0.078	0.077	0.070	0.056	0.079	0.274	0.209	0.200	0.103
$P < 0.01$	0.057	0.105	0.103	0.094	0.075	0.106	0.367	0.280	0.268	0.138
$P < 0.001$	0.063	0.117	0.115	0.105	0.084	0.118	0.411	0.313	0.300	0.154

The signs of leads are not indicated in the table

Table IV

Analysis of variance of alterations in mean values of the T-wave

Variable		SQ	FG	MQ	F
Lead I	Total	0.081	54		
	Treat (between days)	0.025	4	0.006	5.58**
	Error	0.056	50	0.001	
Lead II	Total	0.353	54		
	Treat (between days)	0.162	4	0.041	10.62***
Lead III	Total	0.274	54		
	Treat (between days)	0.090	4	0.023	6.11***
	Error	0.184	50	0.004	
Lead aVR	Total	0.230	54		
	Treat (between days)	0.077	4	0.019	6.29***
	Error	0.153	50	0.003	
Lead aVL	Total	0.146	54		
	Treat (between days)	0.048	4	0.012	6.12***
	Error	0.098	50	0.002	
Lead aVF	Total	0.295	54		
	Treat (between days)	0.101	4	0.025	6.51***
	Error	0.194	50	0.004	
Lead CV_6LU	Total	2.538	54		
	Treat (between days)	0.195	4	0.049	1.04
	Error	2.343	50	0.047	
Lead CV_6LL	Total	1.554	54		
	Treat (between days)	0.191	4	0.048	1.75
	Error	1.363	50	0.027	
Lead CV_5RL	Total	1.458	54		
	Treat (between days)	0.205	4	0.051	2.04
	Error	1.253	50	0.025	
Lead V_{10}	Total	0.348	54		
	Treat (between days)	0.017	4	0.004	0.64
	Error	0.331	50	0.007	

serum, U-waves were found already on day 2 in 7 dogs on 31 occasions in the various leads; on day 3, U-waves were observed in 10 dogs on 52 occasions, whereas on day 4 in 10 dogs on 48 occasions. On day 5, although the serum potassium level continued to decrease, the occurrence of the U-wave tended to decrease. Of the leads used, this pathological wave was most frequently encountered in the chest leads and in lead aVR (Table V).

Discussion

In dogs, the S–T segment and the T-wave may deviate from the isoelectric line in both directions (elevation, depression) and their regular contours may also undergo changes. The S–T segment can be considered normal if it coincides with the isoelectric line, or deviates from the latter only slightly in convex or concave direction. In dogs, the polarity of the S–T segment and T-wave is

Table V

Incidence of the U-wave in the various leads during the 5-day period of study

Lead Day	I	II	III	aVR	aVL	aVF	CV_6LU	CV_6LL	CV_5RL	V_{1e}	Total	Number of dogs with U-wave incidence
1												
2	3	2	2	3	2	1	5	6	6	1	31	7
3	5	4	2	7	2	4	8	8	8	4	52	10
4	4	4	3	4	5	4	7	9	7	1	48	10
5	2	2	5	4	2	2	6	7	8	2	40	9
Total	14	12	12	18	11	11	26	30	29	8	171	

variable and may not be identical with that of the QRS complex, as opposed to human ECG (Ettinger and Suter, 1970; Hilwig, 1976). It follows from this fact that the rules formed on the basis of alterations in the S–T segment and T-wave of the human ECG may not be applicable for the ECG of carnivores. In dogs, the T-wave is not easy to interpret, since it can be positive, diphasic or negative even under physiological conditions. In studying ventricular repolarization, the most reliable results are obtained if the ECG is compared with earlier ECGs of the same animal, during a period of several days.

During the 5-day period of study the quantity of extracellular K^+ significantly decreased upon the effect of the cation-exchanger and the diuretic drug, while the intracellular K^+ remained unchanged. Due to the developed hypokalaemia, the ventricular repolarization stage of the ECG showed signs deviating from the physiological, proportionally to the decrease of the serum potassium concentration.

Deviations of the S–T segment became pronounced in the various leads of all dogs but one, and were observed in particularly high numbers in leads II, aVF and III.

As compared to baseline values, the amplitude of the T-wave decreased significantly in the standard leads and moderately in the chest leads. This is in agreement with the observations of Collet (1982), who considered the low and diphasic T-wave to be signs characteristic of hypokalaemia. Coulter et al. (1975) experimentally induced hypokalaemia in 6 dogs in halothane and pentobarbital anaesthesia, in dorsal recumbency by a single intravenous injection of isotonic NaCl and, although they examined only lead II, interpreted the depression of the S–T segment and the large positive T-wave as typical signs of hypokalaemia. These authors failed to observe U-wave, although, together with Ono et al. and Seta et al. (cited by Coulter et al., 1975), they regard it as a sign typical of hypokalaemia.

In hypokalaemia, Kaemmerer (1975) observed a slight increase of the T-wave in the standard leads, while a decrease thereof in the chest leads. He

also considered the appearance of the U-wave characteristic of hypokalaemia. According to his studies, the U-wave appeared at serum potassium concentrations of 3.8 mmol/l, 3.6 mmol/l and 3.0 mmol/l in 64%, 71%, and 78% of the dogs, respectively. The U-wave occurred most frequently in the three chest leads and in leads aVR and II.

According to Musselman and Hartsfield (1976), hypokalaemia may not be accompanied by striking ECG signs; therefore, all leads should be examined thoroughly. The same authors regarded the U-wave appearing after the T-wave or merging with the latter as the most certain indication of hypokalaemia.

In man, after administration of drugs against angina pectoris, U-wave alterations show the opposite trend to changes found in the magnitude of the T-wave; this can be interpreted as an effect counterbalancing the slowing-down of repolarization. When repolarization slows down, K^+ reabsorption may last even during the U-wave (negative post-potential; Széplaki, 1980).

In the present studies, the incidence of U-wave was proportional to the decrease of serum potassium concentration both in the experimental animals and in the various leads. Appearance of the U-wave in dogs is always a pathological phenomenon, and, together with other signs indicative of ventricular repolarization disorders (S–T segment deviations, alterations in the amplitude of the T-wave), may be interpreted as an ECG sign characteristic of hypokalaemia.

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BOOK REVIEWS

ROLLE/MAYR: Medizinische Mikrobiologie, Infektions- und Seuchenlehre. Editor: Anton MAYR. Ferdinand Enke Verlag, Stuttgart, 1984. 5th edition. 1030 pp., 209 figs, 139 tables. (In German.)

Contributors *(P. A. Bachmann, B. Gedek, H. Mahnel, A. Mayr* and *H. Schels)* to this new revised and partly enlarged edition explain the scope of the book to serve the need of veterinarians, biologists and agronomists, including students. The book covers mainly topics on the pathogens of domestic animals, but short references are made also to infectious diseases of man and lower animals.

The book is composed of three main parts: 1. general epidemiology (142 pp), 2. virology (450 pp), and 3. bacteriology and mycology (403 pp).

General epidemiology covers definitions and explanations on the mechanism of infection and immunity on the broad lines of the former edition. The new concept of "paramunity" as "increase of nonspecific protection of the organism by medicinal means" formulated by the editor *(A. Mayr)* in recent years has been introduced. Theoretical details of the concept are elaborated in the introductory part of the work. Subsequently, "paraspecific" treatment of infectious factor diseases, according to this concept received attention throughout the text of the book.

The second part covers virology and virus diseases. The part on general virology is a concise, well-documented and illustrated compilation on our present knowledge about the morphology, biology and classification of animal viruses. Chapters on virus diagnosis, vaccines and therapeutic agents contain most useful information also for the practician. A short summary each deals with essentials on viruses of fish, reptiles, insects, monocellular organisms and plants as well.

The information on animal virus diseases is arranged according to the last rules of virus classification and taxonomy. In case of individual diseases, a short historical review is followed by paragraphs on the aetiology, epidemiology, pathogenesis, pathology, clinics, diagnosis, immunity and control of the disease. In the list of selected literature added to the chapters, priority for more recent publications instead of historical ones would assist those interested in further references on the subject. A few inaccuracies in the text do not affect the authors having succeeded in producing an excellent up-to-date and concise, though easily readable, presentation about the most important virus diseases of animals.

The part on general bacteriology and mycology describes the structure and function of the bacterial and fungus cells, including their metabolism and the classification of pathogenic germs. It seems unusual to have mixed general bacteriology and mycology, probably for conciseness and comparison purposes. In some paragraphs, such as the genetics of these germs, there is a clear preference for details on bacteria over fungi. In respect of bacterial systematics, the reason for giving tables on both the 1957 and 1974 systems (7th and 8th edition of Bergey's manual, respectively) is not understood. It clearly urges for a new edition for this invaluable work. It should be acknowledged, however, that new pathogenic species have been included with the special part of this section, too. Some uncertainty is felt with chapters such as "infectious factor diseases with Pasteurellae", or "mixed *Clostridium perfringens* infections". This might account for finding the heading "enzootic pneumonia of pigs" twice (pp. 811 and 942) for diseases of different aetiology. Data on the occurrence of some diseases (e.g. meloidosis, CBPP) and the original literature quoted, point to a relatively long processing period of these parts of the work.

The undertaking to deal in one concise volume with the whole subjects of microbiology including virology, immunology and the special pathology and control of viral, bacterial and mycotic diseases of animals seems to be an undertaking ever more difficult to master. Nevertheless, most parts of the book will be a useful source of information for both students and practitioners interested in the broad subject of infectious diseases of animals. The publisher should be complimented for the high standard of production.

T. SZENT-IVÁNYI

RALOVICH, Béla: *Listeriosis Research – Present Situation and Perspectives.* Akadémiai Kiadó (Publishing House of the Hungarian Academy of Sciences), Budapest, 1984. In English language, 222 pages.

The author has dedicated this monograph to Professor Seliger, one of the most outstanding personalities in current listeriosis research, who wrote a preface to the book. In the introduction, the author offers a brief historical survey of the listeriosis problem, then discusses the *bacteriology* (taxonomical problems, biological properties, serotyping, nutrient requirement, ultrastructure, significance of lipids, modes of culturing, isolation and typing, with particular respect to phage typing, as well as the L forms) of *Listeria monocytogenes*, in the light of the most recent data of the literature.

In the chapter *Pathogenicity and virulence* the author touches upon questions associated with haemolysin production and lipase activity, differences between virulent and avirulent strains in cell membrane structure, the monocytosis-inducing agent, and other (e.g. tumour-inhibiting) properties. Finally, data are given on the genetic regulation of certain biological properties.

In the chapter entitled *Epidemiological and clinical manifestations of listeriosis*, the percentile proportion of bacterium excretors on a world scale, listeria-positive test materials, mode and seasonality of infection, relationship of human and animal listerioses by serotype, predisposing factors, and therapy of human listeriosis are illustrated in Tables. As regards prevention, in humans the necessity of a strict observation of food-hygienic regulations, while in animals that of active immunization is stressed.

The *Appendix*, consisting of 14 pages, gives detailed instructions and receipts of media for the pretreatment of test materials and selective culturing and identification of Listeria.

The monograph contains *897 references*; thus, it is a valuable aid for researchers who want to get a more thorough knowledge of any field of listeriosis research. At the end of the monograph there are four histological pictures illustrating intracellular Listeria organisms in the epithelial cells of an infected conjunctiva.

The English translation is easy to understand; however, the use of the word "*oxen*" instead of "cattle" or "cow" is a disturbing mistranslation which results in absurd distortions of the sense, e. g. on page 73: ". . . 0.8 to 5.3% of the oxen excreted Listeria in milk". It would have been good if a veterinarian proficient in English had supervised the translation. Disregarding this smile-provoking flaw, the monograph contains the most recent data and up-to-date knowledge. Thus, it serves as a good starting point for future research aimed at a better understanding of the listeriosis problem, which is insufficiently known even at present.

F. KEMENES

HORZINEK, Marian C.: *Kompendium der allgemeinen Virologie.* Pareys Studientexte, Band Nr. 4. 2nd (revised) edition, 1985. 159 pages, 86 figures and 16 tables. Balacron br. DM 29,–.

All honour to the scientists who are able to impart their knowledge, experience, and thoughts in a logically systematized form, in concise and clear style, allowing the readers to get an overall picture of the discipline in question. The author of this Compendium (pocket-manual), Professor M. C. Horzinek, has met the above requirement; his manual could serve as an example for other branches of science.

The Compendium introduces the subject of general virology and its principles through animal viruses; the properties of the viruses of plants and bacteria are dealt with only when deemed necessary from the viewpoint of comparative virology.

The pocket-manual consists of four main chapters. The first chapter, constituting half of the size, gives a concise account of virological methods, morphological properties of virions, as well as of the taxonomy of viruses. The second chapter summarizes the knowledge concerning infectivity and genetics of viruses, while the third and fourth chapters the facts necessary

for a better understanding of the role of viruses in the aetiology of diseases and in disease outbreaks.

The Compendium offers a full review of general virological knowledge; thus, it can be used by specialists with different levels of training. It helps students understand the material better and prepare for their examinations, and offers a broader knowledge of the general principles of virology as a whole for those virologists and people dealing with related branches of science who have acquired the mastery of any narrower field of virology. The pocket-manual can be of use for qualified assistants working in virological or biological laboratories.

A great advantage of the Compendium is that, as regards the details of methodology, it refers to special manuals offering more detailed information on the subject. The same holds true for certain parts of all the four chapters. Thus, naturally, the Compendium cannot meet all requirements of specialists working in a narrow special field of virology. It must not be forgotten that this pocket-manual is not a text-book, only a guideline which requires from the Reader a certain level of qualification.

Ten years have elapsed since the publication of the first edition, during which time virology has made considerable advances. The author has completed the second edition with this new body of knowledge; i.e. the essence of up-to-date molecular-biological methods by which the nature of substances constituting the viruses (proteins and nucleic acids) as well as the role of these substances in determining the properties of viruses, have been elucidated. The present situation of virus genetics and its role in the strategy of viral replication, pathogenesis of diseases, variability of viruses and aetiology of tumours are also reviewed, as well as the concept of the recently discovered viroids and prions. In virus classification, the Compendium follows the latest standpoint of the ICTV. The decimal classification used in the second edition makes the Compendium more easy to survey and read. The fact that, as compared to the first edition, the size of the second edition has not increased although it contains the new body of knowledge reflecting the advances in virology, deserves special credit.

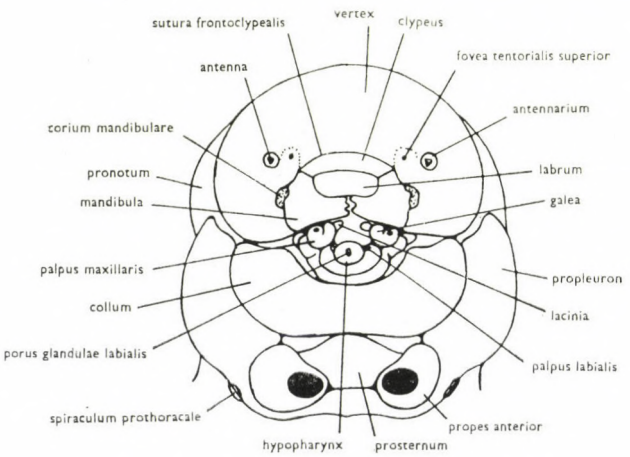
This Compendium, written in German language, can be warmly recommended for specialists, physicians, veterinarians, biologists, agricultural engineers, qualified assistants, and students dealing with, or working in, any field of virology. An English-language edition of the pocket-manual would considerably increase its reading public.

A. BARTHA

A MORPHOLOGICAL ATLAS OF INSECT LARVAE

by H. Steinmann and L. Zombori

In English · 1984 · 404 pages · 1069 figures · 17×25 cm
Hardcover $46.00/DM 117.—/£27.50
ISBN 963 05 3417 7



Comprehensive works discussing the morphology of the insect larvae are very scarce. A general atlas showing the postembryonic forms of the insects, although it would be of great importance in comparative morphology, has been non-existent this far.

The treatment follows the two large natural units the hemi- and holometabolous insects. Each item is preceded by a simplified homologized set of figures, then the groups are treated in the sequence of the insect orders. Newly designed or adapted illustrations constitute the major part of the book showing an advanced stage of larval development.

The figures are numbered consecutively and for the purpose of identification the legends are given in Latin. The atlas proper is followed by a Latin—English and an English—Latin index. A selected reference list closes the book.

AKADÉMIAI KIADÓ, BUDAPEST

AN ATLAS OF INSECT MORPGOLOGY

by H. Steinmann and L. Zombori

In English · Second, revised edition, 1984 · 253 pages · 756 figures · 17×25 cm
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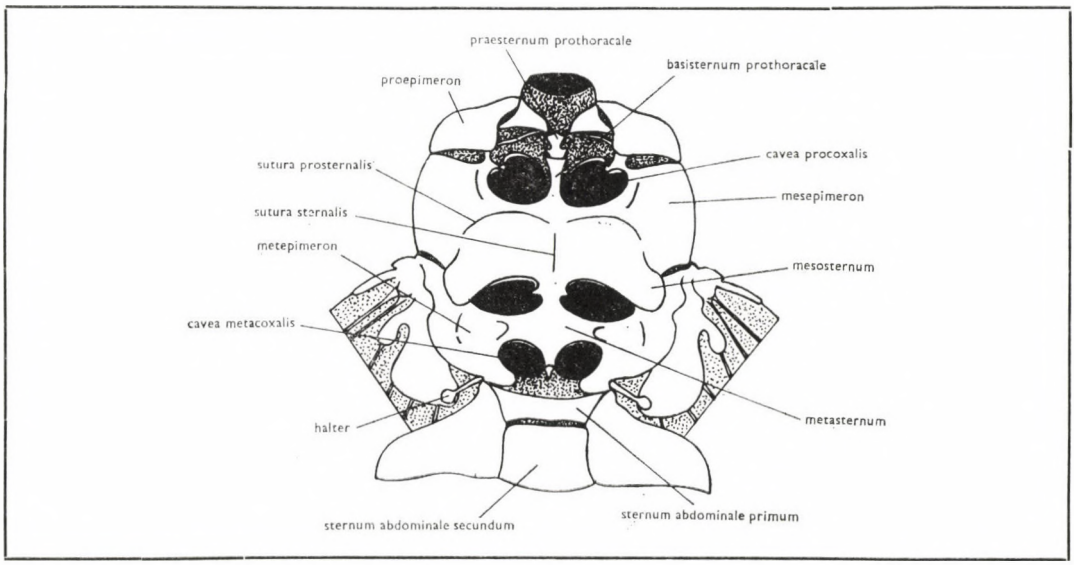

The lack of descriptive works treating the external morphology of insects, as well as the want of a general morphological atlas with a unified terminology induced the authors to compile, on the basis of extensive homology, their general atlas of insect morphology. Accordingly, all was unified that was common in the external skeletal elements of this group of animals, aiming thereby to provide a basic terminology shown on a wide selection of illustrations. The encouraging international reception has stimulated the authors to revise the first edition by carefully considering the constructive and valuable suggestions of many reviews.

The main part or the book is the atlas proper grouping the material according to head, thorax and abdomen including appendages and genital organs. For the second edition some figures have been newly designed to replace earlier ones, also several entirely new figures have been included. The figures are supplied with Latin inscriptions and numbered consecutively. With the new figures scores of new terms have been added to elucidate the nomenclature of various bodily parts. The figure captions are English. The atlas is provided with a Latin-English and English-Latin index for the figures. A list of selected references provides a lead to those fundamental handbooks or other parts that were our sources.

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