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TRADITIONAL AND NEW CONCEPTS IN PROTEIN EVALUATION OF FEEDS: A REVIEW

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The purpose of protein evaluation of a diet is to determine its value for meeting amino acid requirements of animals. The early recognition that proteins can be utilized by different efficiency rates has promoted extended research to reveal quality differences and their reasons. A brief historical overview is given in this review about the development of concepts and theories in protein evaluation.

Recent developments have focused attention on determination of available quantities of essential amino acids. *In vitro* chemical assays and dye-binding procedures are relatively fast and provide data without access to animals, however they do not always correlate well with biological estimates. Enzymic digestion methods and microbiological assays for prediction of availability of essential amino acids have the potential for serial determination in quality control, however they are constrained by a lack of information about the availability of peptides liberated and utilized during *in vivo* digestion of proteins.

Animal growth assays and balance experiments can provide valuable estimates of available quantities of essential amino acids. However, the interpretation of data is complicated by the many factors affecting *in vivo* protein utilization, by the endogenous amino acid excretion and the effects of microflora of the alimentary tract.

The search for reliable assays remains a worthwhile objective also in the future.

Keywords: Protein quality, assay methodology, availability, feedstuffs, review

The nutritive value of a feed protein depends primarily on its capacity to satisfy the needs for amino acids of animals. Different protein sources may have different nutritive values depending on their ability to provide essential amino acids for various biochemical functions of the organism. As the *essential amino acid requirements may vary* according to age (young or grown-up animal), physiological state (maintenance, growth, repletion, pregnancy, lactation etc.) and type of production (meat, milk, egg etc.), the same protein consequently may have different nutritive values, depending on the criterion used for its evaluation.

A brief historical overview

The recognition that nitrogen-containing compounds are essential in nutrition dates back to the first decades of the nineteenth century (Magendie, 1816). Liebig (1846), in order to distinguish proteins from energy-supporting fats and carbohydrates, called them "plastic" elements. However, he believed

that any kind of protein had the same nutritive value, and this way the mere determination of nitrogen content was assumed to be satisfactory for the evaluation of proteins in foods or feeds.

In the early stage of nutrition sciences the view was mainly quantitative. This may be illustrated by Rubner's "law of isodynamics" which assumed that nutrients can equivalently substitute each other by their energy contents. By the turn of the last century, knowledge has considerably expanded on the diverse quality of dietary proteins. Before identifying all amino acids commonly found in proteins, results of nutritional experiments suggested that proteins of different origin may differ in nutritive value.

Voit's (1872) research on the widespread use of gelatine in nutrition showed that glue cannot replace meat proteins in dogs. As proteins of different origin could not substitute each other by weight, Rubner (1897) came to the conclusion that different kinds of proteins must have different biological values i.e. they are of different quality. The reason for these differences was not understood until Willcock and Hopkins (1906) found that lack of certain amino acids was responsible for such inadequacies. It became more and more likely that the nutritional quality of proteins depended on amino acid composition.

The discovery of amino acids commonly found in proteins started with the identification of glycine in 1890, and finished with the isolation of methionine in 1922 and threonine in 1935 (Mitchell, 1952).

The quantitative characterization of proteins involved first the preparation of pure isolates from biologic materials to remove interfering substances. A significant development in these techniques has been achieved as early as in the first decades of this century.

The application of microbiologic methods, developed originally for assaying vitamins, for the quantitative estimation of amino acids in the 'forties and 'fifties has greatly promoted the accumulation of data on amino acid composition of many different proteins.

Evidences of many experiments have shown that the nutritive value of proteins was largely dependent on the proportions of amino acids resulting from their hydrolysis. Some amino acids proved to be essential to support growth (lysine, methionine, tryptophan, threonine, phenylalanine, valine, leucine and isoleucine), while others did not. The relationships between tyrosine and phenylalanine, as well as cystine and methionine, were soon recognized. Cystine and tyrosine appeared to be essential only if the diet contained inadequately low levels of methionine and phenylalanine, respectively. Histidine, arginine and glycine were found "semi-essential", because they could be synthesized by the animal at rates not adequate for maximum growth.

This classification enabled authors to focus attention on essential amino acids. The essential amino acid content of the whole egg proved to be nearly

completely available for rats. By comparing the essential amino acid contents of a protein with those of whole egg, Mitchell and Block (1946) computed the percentage deviations for each amino acid.

Systematic biological evaluation of proteins may be said to have begun in the first decade of this century.

Folin (1905) introduced the term of "dichotomy of protein metabolism" in 1905. According to this concept protein metabolism may be divided into two independent phases: the *endogenous phase* is constant and pertains to the irreversible erosion of tissues; the *exogenous phase* is variable, being dependent in rate on the protein intake. The term "dichotomy" later proved to be very fruitful in evaluating proteins. This theory of protein metabolism is the fundamental base of the methods measuring nitrogen balance even today.

Rubner's view concerning minimum human protein requirement arose Thomas's interest. Using himself as an experimental subject, in 1909 Thomas worked out a method based on measuring the nitrogen balance of the body. This method was based on the observation that the nitrogen balance of the organism can be set by different quantities of proteins, depending on their origin. In his work, considered to be a classic today, the biological value of proteins was defined as the retained portion of absorbed nitrogen expressed in per cent. His formula, which included the correction for "endogenous" and "metabolic" faeces nitrogen losses as well, has stimulated worldwide scientific interest.

Classical terms of evaluating dietary proteins which are used even today were established on the basis of Thomas's work.

Thomas's results had a great impression on Mitchell. The method for assaying biological value of proteins in humans was adapted by him for growing rats. Research in Mitchell's American laboratory and in numerous European institutions has made the method popular and it has become known as the "Thomas—Mitchell method" for assessing the biological value of proteins. In a modified form (Mitchell, 1924) it is still the basis of measuring the biological value of proteins.

Systematic experiments with Thomas's method showed that the biological value of each protein is different and animal proteins are usually more valuable than those of plant origin. Explanation for these differences became possible only much later, after the discovery and isolation of all amino acids.

Osborne's and Mendel's fundamental studies also led to the recognition that the nutritive value of proteins depends on their amino acid content and on relative rates of amino acids.

The first detailed studies on the quality of proteins were also conducted by Osborne and Mendel between 1911 and 1920 (Mitchell, 1952). Osborne and Mendel, isolating and analysing plant proteins, examined how these proteins were able to support the growth of rats. On the basis of their effect on growth,

they divided proteins into two groups: "complete" and "incomplete" proteins. Later on a more sophisticated experimental method for expressing numerically the growth-promoting value of proteins was developed. Weight gain was related to the quantity of protein intake, and thus the efficiency of different protein sources was measured (Osborne et al., 1919).

McCollum (1914), taking into account demands of livestock production, conducted practical nitrogen balance experiments on growing pigs. He expressed the results as percentages of the ingested nitrogen absorbed (corrected for metabolic faecal nitrogen) and percentages of absorbed nitrogen recovered in the nitrogen balance.

The principle of the Thomas method has been applied not only to growing rats, but also to pigs, sheep, poultry and to man. The biologic values of dietary protein, expressed as the retained part of ingested protein, may vary theoretically from 0 to 100. However, values less than about 40 seemed to be incompatible with growth in rats. The nutritive values of proteins for maintenance of nitrogen balance have been shown to be different from those for growth. The low quality proteins proved to be more efficient for replacement of endogenous losses of nitrogen than for supporting growth.

The so-called "regeneration" methods represent an independent branch of dietary protein evaluation. It has been emphasized that different protein sources and protein fractions may contribute to the regeneration of various tissues to a different degree. Accordingly, the relative sequence of proteins may be divergent, therefore these methods can answer only specific questions (Melnick et al., 1936; Chow et al., 1948; Davis and Whipple, 1921; Harrison and Long, 1945; Henry et al., 1953).

Examination of tissue enzymes is considered to be a logical step following the spread of methods based on regeneration of tissue proteins. These methods examined how the activity of an enzyme changes depending on the quality of dietary proteins. An example of the methods is testing the activity of xanthine oxidase of the liver: a deficiency of essential amino acids of the dietary protein reduces the rate of oxidation of xanthine into uric acid (Bavetta and Narrod, 1957).

It is known today that native proteins usually consist of about 20 amino acids. Eight to 12 of them is regarded to be "essential" depending on the species and physiological state of the animal. These cannot be synthetized by the organism in proper quantities, therefore they must be included in the diet.

As it is confirmed by numerous experiments, the nutritive value of proteins is related to their essential amino acid content. These experiences have established a new attitude in nutrition sciences. Priority has been given to required values of amino acids instead of protein requirements. The expanding knowledge of amino acid composition of feeds and of essential amino acid

requirements of farm animals led to the possibility of fulfilling the nutritional requirements by least-cost linear programming.

The new liquid chromatographic method developed by Stein and Moore has promoted collecting data on amino acid composition of feed proteins. The method was automatized in 1958. Today automatic amino acid analysers are widely used in many laboratories (Spackman et al., 1958).

The "limiting essential amino acid" concept of Block and Mitchell (1946) represented great progress in prediction of biological quality by amino acid scores. The values of each essential amino acid of a test protein were individually expressed in percentage of the content of a corresponding amino acid in a suitable reference amino acid pattern. The lowest proportion was claimed to limit the protein utilization, and is called "chemical score" or "score". Later, different multiple regression equations and various mathematical models were developed to predict biological indices of protein quality from amino acid data, as reviewed recently by Hidvégi and Békés (1985).

The experiences of Kofrányi and Müller-Wecker (1961) suggested that the utilization of dietary protein is also affected by nonessential amino acids which always occur in proteins. The nonessential amino acids might have a sparing effect on essential amino acids. Impressed by the results of many experiments with protein mixtures on adult humans, Kofrányi (1970) at last seriously questioned the overall validity of the concept of "limiting essential amino acids". However, the supplementation of feed proteins with their first limiting essential amino acids generally results in a significantly better utilization of dietary protein in growing animals.

The international nutrition programmes of the UN, WHO and FAO had a great stimulating effect on research of protein evaluation. To meet the energy-protein requirements of the undernourished population of developing countries and for a more economical and efficient feeding of farm animals, there was an urgent need for exploring and qualifying unconventional protein sources.

In the last two decades there has been considerable interest in monitoring protein quality in feeds, not only to define *nutritional requirements* of farm animals but to provide *standards* of quality for commercial food and feed products. Great interest has also arisen in *screening* for protein quality in the production of food or feed crops, to produce improved varieties of cereals (corn, wheat etc.), legumes (beans, peas etc.) and roots (potatoes etc.).

The nutritional value of dietary protein, as explained earlier, depends primarily upon the *amount* and *patterns* of its *essential amino acids*. Therefore the comprehensive evaluation of protein quality must consist, first of all, of the determination of *nitrogen* content, *amino acid* composition, and *digestibility*. Finally, the *capacity* of proteins to fulfil nutritional needs is to be predicted.

The concepts of evaluating protein quality are closely related to the theories of protein metabolism. Parallel with the progress in methodology

— especially in balance studies — different models were published (Miller and Payne, 1963; Gebhardt, 1966; Rufeger, 1972; Mercer et al., 1985; Krawielitzki and Bock, 1976). These have been subjects of scientific debates.

During the past decades considerable efforts have been devoted *to develop fast and relatively simple in vitro and in vivo methods* which are useful in screening large numbers of samples and are able to monitor, explore or predict the nutritive value of dietary proteins. The methods for assessing *available* quantities of essential amino acids are the most promising from this respect.

Determination of available quantities of essential amino acids

The nutritive value of proteins may be estimated by the total amino acid content determined by chemical or microbiological methods. However, in the case of heat-processed proteins the nutritive value of proteins may be much lower than that expected from the total amino acid content. This indicates that the total quantity of amino acids is not fully "available" for the animal for metabolic purposes.

Availability is an abstract concept. The available amino acid content of the dietary protein mainly depends on the quantity of amino acids absorbed in the course of digestion. The absorption is a prerequisite for, but not proof of, availability. The absorption rate of amino acids may be affected by the composition of the diet (fibre, tannins etc.), and physiological characteristics of the animal (e.g. the passage rate of nutrients in the lumen of the digestive tract). Only absorbed (digested) amino acids may be available, however certain amino acid derivatives are excreted via the urine without being utilized for protein synthesis (e.g. fructosyl-lysine).

The amino acid quantity which serves for meeting the energy requirements of the animal is mostly lost for protein synthesis. Therefore only that quantity of amino acids may be regarded as available which takes part in protein anabolism. This quantity cannot be measured directly but it can be estimated by many different methods.

From a practical point of view, the *true digestible amino acid content determined by faecal method* is often considered to be analogous with the available amino acid content. Significant difference between digestible and available amino acid content mainly occurs when heat-processed protein is used for feeding.

In feeding practice, information on the available quantities of *lysine*, *methionine* (methionine + cystine), *tryptophan* and *threonine* is of great importance as usually these four amino acids occur in the lowest proportion in industrial feed mixtures, as compared to the requirements of animals.

For estimating available amino acid content of feeds ideally animal assays should be applied. However, *in vitro* methods having a close correlation with values of animal experiments may also be appropriate.

Chemical methods

Chemical methods which are widely used at present were developed only for measuring available lysine and methionine.

Lysine having free and thus reactive epsilon amino group is considered to be available to the animal. Several assays have been developed to measure reactive and unreactive lysine in proteins. These depend on the reaction of chemically accessible epsilon amino groups with *1-fluoro-2,4-dinitrobenzene* (FDNB; Carpenter, 1960); *2,4,6-trinitrobenzene-sulfonic acid* (TNBS; Kakade and Liener, 1969); *O-methyl-isourea* (OMI; Mauron, 1966); *conjugated vinyl compounds*, such as *methyl acrylate* (MA), *methyl-vinyl-sulfone* (MVS), *ethyl-vinyl-sulfone* (EVS), *phenyl-vinyl-sulfone* (PVS; Friedman and Finley, 1975); *S-ethyl-trifluoro-thioacetate* (Ramirez et al., 1975); *acid dyes* (Hurrel et al., 1979) *and ninhydrin* (Friedman and Williams, 1972). These methods have been extensively reviewed recently by Friedman (1982).

Methionine and *methionine sulfoxide* are equally available to rat and chicken, but the more oxidized *methionine sulfone* was shown to be completely unavailable. An early colorimetric method for differentiating between methionine and its oxidized forms was developed by McCarthy and Sullivan (1941). A more recent method (Lipton and Bodwell, 1977) depends on the reducing effect of unoxidized methionine on *dimethyl sulfoxide* converting it to *dimethyl sulfide*. The latter can be measured by GLC techniques. Unoxidized methionine content of an intact protein is able to react with *CNBr* to form *methyl thiocyanate* (MeSCN), which can be measured by gas chromatography (Smith, 1972; Dévényi et al., 1974; Váradi et al., 1976).

An automated colorimetric method for determination of *unoxidized methionine* using *iodoplatinate* reagent was described by Njaa (1980). Methionine sulfoxide does not react under the chosen conditions. The method may be used to distinguish between unoxidized and total methionine without and with previous reduction of the sample with titanium trichloride. Methionine sulfoxide is then obtained by the difference.

The bioavailability of *L-cystine* depends on the degree of its oxidation. Determined by chicken bioassay (Crawford et al., 1984) *cystine monoxide and symmetrical cystine disulfoxide* were shown to be nearly as available as L-cystine. However, *unsymmetrical cystine dioxide* was found to be only about half as available as *L-cystine*. *L-cystine sulfonate* (cystein sulfonic acid or cysteic acid) was completely unavailable for the growth of chicks.

For measuring available cysteine in processed feeds (e.g. feather meal) *dithiothreitol* (DTT; Clelands reagent) was used to convert all cystine to cysteine and the quantity of cysteine was measured by a colorimetric method (Pienjazek et al., 1975). Cysteic acid does not yield colour under the conditions used, therefore only the available cysteine is measured.

In animal growth assays to determine bioavailable cystine *chicks* are preferred, as *rats* and *pigs* have a very low specific requirement for cystine (Eggum and Pedersen, 1983).

Dye-binding procedures

Azo dyes react with the free epsilon-amino groups of lysine, histidine and arginine, as well as with the terminal amino groups of the peptide chain. Measuring *dye-binding capacity* (DBC) of samples with Acid Orange 12 before and after treatment with propionic acid anhydride or acetic acid anhydride the reactive lysine (dye-binding lysine, DBL) content may be determined (Hurrell et al., 1979).

Chemical assays and *dye-binding* procedure data do not always correlate with biological estimates of available lysine. However, these assays may be useful in quality control, to make comparisons among samples within ingredients. Dye-binding capacities of heat-treated proteins may also be used for monitoring early *Maillard* reactions together with the measurement of soluble saccharides having free oxo groups (Molnár-Perl and Pintér-Szakács, 1986).

In vitro enzymatic methods

Measuring the content of each amino acid before and after *in vitro* enzymatic hydrolysis of a protein offers the possibility to evaluate their digestibility and availability. Methods of this type may be used in combination with dialysis (Savoie and Gauthier, 1986) or ultrafiltration (Floridi and Fidanza, 1975). *In vitro* enzymatic methods have been reviewed comprehensively by several authors (Mauron, 1970; Stahmann and Woldegiorgis, 1975; Hegedüs et al., 1981).

Microbiological methods

Streptococcus zymogenes (Ford, 1962) and *Tetrahymena pyriformis W* (Shorrock, 1976) were introduced as test organisms for measuring the available quantities of essential amino acids. Growth of the test microorganisms in appropriate nutritive media is limited by the available quantity of the amino acid to be determined. The principle of the method is similar to that of the assays based on animal growth.

The absolute values of microbiological assays are affected by several variables, and therefore they can serve primarily for ranking proteins, or for quality control of different batches of feed ingredients.

The accurate measurement of protozoan growth is complicated (Hegedüs, 1973), therefore bacterial assays may be preferable.

Monogastric animals may utilize peptides as sources of amino acids better than do microbes.

Animal growth assays

The growth of experimental animals (rats, chicks) is proportional to the available quantity of the limiting essential amino acid of the protein tested. The basal diet, which can be of synthetic, semisynthetic or natural origin must be deficient in the amino acid to be assayed.

To obtain a standard dose-response relationship, the basal diet is supplemented with graded amounts of the lacking amino acid. If a protein is mixed with the basal diet the growth response of the animal, compared to the standard, indicates the available quantity of the limiting amino acid. This value expressed in percentage of the total content of the same amino acid determined by chemical analysis yields the figure of availability.

As the growth of the animal depends also on the protein level in the diet, *isonitrogenous* levels must be used or the values are to be extrapolated to zero protein level using graded amounts of test proteins.

These methods are based on the principle of the determination of essential growth factors (e.g. assessment of B vitamins by microbiological assays), therefore are only applicable to evaluate availability of amino acids which are essential.

Various methods based on the growth of young animals (rat, chick, pig) have been developed for the evaluation of availability of lysine and methionine (e.g. Calhoun et al., 1960; de Muelenaere et al., 1967; Hill et al., 1966; Carpenter et al., 1963; Opstvedt and Mundheim, 1977; Combs et al., 1968; Guttridge et al., 1961; Miller et al., 1965; Sasse and Baker, 1973; Abbey et al., 1980; Donnelly and Rattray, 1983).

Concepts, criteria used for evaluation, and techniques of methods have been comprehensively reviewed by several authors (e.g. Guttridge, 1962; Erbersdobler, 1976; Pellett and Young, 1980; Sibbald, 1987).

The preferred independent variable in animal growth assay is *amino acid intake*, whereas *body weight gain* is most commonly used as response criterion.

Methods for determination of in vivo digestibility of amino acids

In vivo digestibility (apparent or true) of the individual amino acids can be measured by indirect balance methods. The *faecal analysis method* developed originally by Kuiken and Lyman (1948) measures the difference between the

amount of each of the amino acids consumed and excreted in the faeces. These apparent digestibility values may be corrected with the endogenous excretion of amino acids, usually determined by analysing the amino acid levels in faeces of fasting animals or fed protein-free diet, to obtain true digestibility values.

The limitation of the faecal digestibility method is that endogenous amino acid losses are dependent on many factors (intake of dry matter and protein, level of crude fibre, possible presence of antinutritive factors etc.). Moreover the amino acid composition of the faeces is largely unaffected by the amino acid pattern of the protein consumed (Bergner et al., 1980). The reason for this is the *intense fermentation* that occurs in the large intestine of rats and pigs. Thus approximately 60 to 70 per cent of the total nitrogen of the faeces is of bacterial origin.

The *ileal digestibility* of amino acids can be measured with surgically modified animals in which the intake and the amount of each amino acid passing through the distal part of the ileum is determined. The advantage of this method is that the confusing effect of microflora in the large intestine can be avoided (Zebrowska, 1973; Varnish and Carpenter, 1971).

The *digestible amino acid content* of the main ingredients of feeds of farm animals has extensively been studied (e.g. Hermann, 1970) and utilized for diet formulation (Wünsche et al., 1977).

Advantages and limitations of methods and the factors influencing amino acid digestibilities have been discussed by several authors (Eggum, 1977; Eggum and Jacobsen, 1976; Zebrowska, 1978; Sauer and Ozimek, 1986; Tanksley and Knabe, 1984).

The true digestibility of amino acids is considered to be synonymous with bioavailability (Sibbald, 1987).

Nylon bag assays

Digestibility of proteins can be measured directly by putting feed samples in permeable nylon bags and placing them in the lumen of the duodenum via a cannula (Petry and Handlos, 1978; Sauer et al., 1983). The small sample size and independence from correction for endogenous nitrogen has recently focused attention to these techniques (e.g. Taverner and Campbell, 1985).

Possible trends in the future

The main purpose of protein evaluation is to reveal protein damages in feedstuffs or to formulate practical diets which are adequate to meet the amino acid requirements of animals. The expanding knowledge on available amino

acid content of feed ingredients will lead to better estimates of quality of feedstuffs for the feed industry. However, a change from total to available amino acids in practical diet formulation needs additional efforts to collect reliable and consistent data. Developing and testing fast and inexpensive *in vitro* techniques to predict *in vivo* amino acid availability of feedstuffs for different animal species are expected to continue also in the near future.

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CHEMICAL COMPOSITION AND VITAMIN B CONTENT OF ABATTOIR BY-PRODUCT MEALS

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Chemical composition of meat-and-bone meals (n = 22), poultry by-product meals (n = 12), blood meals (n = 6), bone meals (n = 4) and feather meals (n = 8) indicated a slight degree of overdrying. Crude fat and ash contents were within the acceptable limits. Mineral elements of bones were determinative in the element pattern of meat-and-bone meals as indicated by the significant correlations between Ca, P and Mg.

Crude protein was in negative regression and correlation with crude ash content ($r = -0.81$; $P < 0.001$). This was more pronounced when the data were expressed in fatless dry matter ($r = -0.94$; $P < 0.001$). Crude protein also showed significant negative correlations with Ca, P and Mg contents, offering the possibility to predict their levels from nitrogen content.

Meat-and-bone meals and poultry by-product meals were relatively rich sources of riboflavin, niacin, pantothenic acid and biotin, whereas their pyridoxine and thiamine contents were low.

Blood meals, bone meals and feather meals were found to be negligible vitamin B sources. Vitamin B and crude protein content of abattoir by-product meals showed uncertain correlations.

Keywords: Chemical composition, abattoir by-product meals, meat-and-bone meal, poultry by-product meal, blood meal, feather meal, vitamins B, mineral elements.

Abattoir by-product meals (meat-and-bone meal, tankage, poultry by-product meal, bone meal, blood meal, feather meal) are widely used as ingredients in mixed feeds although their nutritive value shows significant variations. The great variance in the chemical composition and growth promoting ability of such products is attributed to the differences in the nature of the raw materials (confiscated organs, blood, feather, poultry legs and heads, guts, bones etc.) and to the conditions used in their processing (Atkinson and Carpenter, 1970*a* and *b;* Eggum, 1970; Kondos and McClymont, 1972; Skurray, 1973; McNaughton et al., 1976).

Standardization of the nutritive value of abattoir by-product meals is difficult, because their raw materials vary in protein and ash content and the quality of soft and hard offals used is also different. On the other hand, the production and use of these products in animal feeds afford possibility for the optimal recycling of slaughter losses, which explains the frequent discussions on the nutritive value of by-product meals (e.g. Sathe et al., 1964; Moran and Summers, 1968; Brüggemann et al., 1969 or, more recently, Fritzpatrick and

Bayley, 1977; Ferrando and Henry, 1977; Skurray, 1982; Hegedüs et al., 1983; Steiner et al., 1983).

To collect data on the composition and nutritive value of abattoir by-product meals, a complex study was supported by the Ministry of Agriculture and Food in Hungary. In this paper, the chemical composition (crude protein, fat, ash, fibre, minerals) and the content of certain B vitamins (thiamine, riboflavin, niacin, panthothenic acid, biotin, pyridoxine) are discussed.

Materials and methods

Origin of samples

The samples of abattoir by-product meals were taken from batches produced on industrial scale in 9 different dry rendering plants. The raw by-products obtained from slaughterhouses were heat treated (130 °C, 30 min), which was followed by the reduction of moisture by drying (100 °C, 4 hours) and lowering the fat content by pressing (Szél and Gál, 1980).

Raw materials of *meat-and-bone meals* consisted of soft offals of varying composition (confiscated organs, blood, rumen and gut with their contents, by-products of fat rendering, inedible parts of swine and cattle), approximately 60% (w/w), and of hard offals (bones, hooves, hair, feather), 40% (w/w).

Poultry by-product meals consisted of all slaughter offals including also feather and intestinal content. The approximate ratios of ingredients were: 10% w/w hatching residues, 50% w/w guts, heads and shanks, and 40% w/w feathers. The meals were produced in 4 different dry rendering plants according to De Vita's technology (De Vita, 1977).

Bone meals were produced from industrial raw bones. *Blood meals* were produced from slaughterhouse flow-off blood in batch rendering cookers. The raw material of feather meals consisted of wet feathers obtained from poultry slaughterhouses. The processing of feathers was carried out without a chemical treatment, only steam pressure (heating) was used.

The commercially available samples were taken from the rendering plants after their microbiological control.

Methods

Crude protein, crude fat (ether extract), ash and crude fibre contents were determined according to the Hungarian Standard MSZ 6830. Mineral levels including sulphur were analysed partly by colorimetry or turbidimetry, partly by atomic absorption spectrophotometry using a Perkin-Elmer type 5000 apparatus, as described by Tölgyesi (1970).

Vitamin B contents (thiamine, riboflavin, niacin, biotin, pantothenic acid, pyridoxine) were determined by microbiological assay with the following test organisms: *Lactobacillus fermenti* ATCC 9338 (thiamine); *L. casei* ATCC 7469 (riboflavin); *L. palantarum* ATCC 8014 (niacin, biotin and pantothenic acid); *Saccharomyces carlsbergensis* ATCC 9080 (pyridoxine). Difco Assay Media and Merck vitamin standards were used. Assay procedures are described in Difco Supplementary Literature (1968).

Results and discussion

Chemical composition

In the Hungarian Standard for animal protein meals (MSZ 21340-86) four *sortiments* of meat-and-bone meals are recommended according to their protein levels (Table I). Poultry by-product meals, blood meals, bone meals and feather meals are considered to have a relatively constant raw material composition, therefore less differentiation was necessary within the same type of meals.

Table I

Recommended chemical composition of abattoir by-product meals*

	Moisture (%, w/w) max.	Crude protein (%, w/w) min.	Crude fat (%, w/w) max.	Crude ash (%, w/w) max.	Calcium (%, w/w) min.	Phosphorus (%, w/w) min.	*In vitro* pepsin digestibility of crude protein (%, min.)
Meat-and-bone meal, sortiment 1	10	62	15	22	—	—	80
Meat-and-bone meal, sortiment 2	10	58	15	28	—	—	80
Meat-and-bone meal, sortiment 3	10	54	15	28	—	—	80
Meat-and-bone meal, sortiment 4	10	50	15	30	—	—	80
Poultry by-product meal, sortiment 1	10	55	10	13	—	—	80
Poultry by-product meal, sortiment 2	10	70	7	11	—	—	73
Blood meal, conventionally dried	10	82	2	6	—	—	80
Blood meal, spray dried	10	86	2	6	—	—	90
Bone meal, from deproteinized bone	10	5	3	—	30	14	—
Bone meal, from raw bone	10	20	12	—	18	9	80
Feather meal, sortiment 1	10	75	6	—	—	—	80
Feather meal, sortiment 2	10	75	8	—	—	—	70

* = Standard for feeding stuffs of animal origin, MSZ 21340 86, Hungary

Table II

Proximate analysis of abattoir by-product meal samples

	Dry matter (g/100 g)	Crude protein (g/100 g)	Crude fat (g/100 g)	Crude ash (g/100 g)	Crude fibre (g/100 g)
Meat-and-bone meals[a] (n = 22)	94.4±2.8	54.9±4.0	11.3±3.2	18.2±4.6	1.5±0.6
Poultry by-product meals[b] (n = 22)	94.6±3.1	65.7±7.2	8.9±3.4	9.3±3.7	1.2±0.4
Blood meals[c] (n = 6)	94.1±3.7	71.8±8.2	3.7±1.1	4.0±1.2	0.0±0.0
Bone meals[d] (n = 4)	97.6±4.1	24.6±4.1	10.3±3.3	59.3±4.7	0.0±0.0
Feather meals[e] (n = 8)	95.1±2.8	81.4±4.5	3.9±1.7	3.8±2.2	0.0±0.0

[a] = Samples were produced in a dry rendering plant. Cooking: 130 °C, 30 min; drying: 100 °C, 4 h. Fat content was partly removed by pressing. Raw materials may contain feathers and gut content;

[b] = Samples were produced from all by-products of poultry slaughtering in a DE VITA dry rendering plant. Cooking: 130 °C, 30 min; drying: 100 °C, 4 h

[c] = Blood was cooked (130 °C, 15 min) and dried at 100 °C for 6 h in a disinfector (autoclave);

[d] = Samples were produced from industrial raw bones. Cooking: 130 °C, 30 min; drying at 100 °C for 4 h;

[e] = Poultry feathers were cooked (130 °C, 45 min) and dried (100 °C, 5 h) in an autoclave

The analysed chemical composition (dry matter, crude protein, ether extract, crude ash, crude fibre and mineral elements) of different commercial abattoir by-product meals is shown in Table II. As compared to the recommended parameters, dry matter contents of all types of meals were higher indicating a certain degree of overdrying. Excessive drying may diminish digestibility and nutritive value. As drying time proceeds, water evaporates and the decrease in the latent heat of evaporation allows the temperature of the meal to increase rapidly (Skurray, 1982). Overdrying to a moisture content of less than 5 per cent enhances the hydrophobic character of the meals and, as a consequence, their digestibility becomes lower and protein damage may occur. The crude fat (ether extract) content of samples was within the acceptable range in all types of meals. As moisture and fat content of meals can be standardized by processing (drying, fat extraction), the moisture and fat levels reflect the preciseness of the technology. On the other hand, protein and ash content of the meals is the consequence of the composition of raw materials.

The *crude protein* content of meat-and-bone meal samples (average = = 54.9 ± 4.0 g/100 g; n = 22) was within the range of recommended values. This applies also to the poultry by-product meals (average = 65.7 ± 7.2 g/100 g). At the same time, however, the average crude protein level of blood meals was lower (71.8 ± 8.2 g/100 g; n = 6) than the recommended values.

Crude ash content of all the samples was lower than the recommended maximum levels. The pattern of mineral elements excluded the presence of ash forming contamination. For the mineral element composition of meat-

Table III

Mineral element composition of abattoir by-product meal samples

	Meat-and-bone meals[a] (n = 22)		Poultry by-product meals[b] (n = 12)		Blood meals[c] (n = 6)		Bone meals[d] (n = 4)		Feather meals[e] (n = 8)	
Macro-elements	mole/kg	cv (%)	mole/kg	cv (%)	mole/kg	cv (%)	mole/kg	cv (%)	mole/kg	cv (%)
K	0.13	40	0.08	33	0.13	20	0.03	10	0.03	10
Na	0.35	25	0.17	25	0.22	4	0.35	13	0.03	14
Ca	1.86	30	0.88	31	0.17	29	7.60	32	0.17	29
P	1.07	27	0.61	21	0.19	33	3.29	40	0.06	5
Mg	0.08	50	0.04	10	0.02	20	0.20	20	0.02	25
S	0.19	17	0.37	17	0.22	14	0.06	5	0.56	22
Micro-elements	mmole/kg	cv (%)	mmole/kg	cv (%)	mmole/kg	cv (%)	mmole/kg	cv (%)	mmole/kg	cv (%)
Al	4.63	39	1.63	27	3.08	45	1.04	29	9.12	40
Fe	26.21	44	32.09	39	46.52	26	4.49	31	24.32	44
Mn	0.49	44	0.58	47	0.18	50	0.11	33	0.47	12
Zn	1.76	17	2.81	27	0.56	51	1.94	6	2.69	29
B	0.18	50	0.28	33	1.02	36	0.09	10	0.18	5
Cu	0.38	38	0.11	43	0.06	25	0.13	13	0.16	1
Mo	0.004	25	0.003	33	0.003	33	0.001	100	0.002	50

cv = coefficient of variation
For footnotes a, b, c, d and e see Table II

and-bone meals (Table III) the element pattern of bone was determinative. The sulphur content of poultry by-product meals (0.37 mole/kg; n = 12) and feather meals (0.56 mole/kg; n = 8) was higher than that of meat-and-bone meals (0.19 mole/kg; n = 22).

The average calcium content of meat-and-bone meals was 1.86 mole/kg (n = 22). When high levels of meat-and-bone meals are used to supplement proteins in cereal diets, the excess of dietary calcium may depress the weight gain in growing chicks (Sathe et al., 1965). However, Atkinson and Carpenter (1970*a*) reported that calcium up to the level of 0.3 mole/kg did not suppress the growth of rats. When supplementation is used as recommended (maximum 10 per cent meat-and-bone meal in the feed), calcium content of meat-and-bone meals does not result in adverse performance.

Crude protein of meat-and-bone meals showed a significant negative correlation with crude ash content, which was more pronounced when the data were calculated in dry fatless matter. This is illustrated by the following equations:

$$y_1 = 67.84 - 0.71x_1;\ r = -0.81,\ (P < 0.001);\ Syx = 2.47$$
$$y_2 = 75.95 - 0.92x_2;\ r = -0.90,\ (P < 0.001);\ Syx = 2.20$$
$$y_3 = 93.51 - 1.25x_3;\ r = -0.94,\ (P < 0.001);\ Syx = 2.48$$

where y = crude protein; x = crude ash; 1 = g/100 g sample; 2 = g/100 g dry matter; 3 = g/100 g fatless dry matter.

Table IV

Vitamin B content of abattoir by-product meals compared with that of some raw materials

	Thiamine-HCl		Riboflavin		Niacin		Ca-panthotenate		Biotin		Pyridoxine-HCl	
	μmole/kg	cv (%)	μmole/kg	cv (%)	μmole/kg	cv (%)	μmole/kg	cv (%)	μmole/kg	cv (%)	μmole/kg	cv (%)
Meat-and-bone meals (n = 12)	0.18	17	11.96	47	290.82	53	9.02	58	1.23	33	4.86	40
Poultry by-product meals (n = 12)	0.12	25	10.63	18	329.81	16	17.84	15	0.82	35	2.92	33
Blood meals (n = 12)	0.24	75	8.77	67	161.66	38	7.97	82	0.12	67	1.95	50
Bone meals (n = 4)	0.12	25	0.80	66	60.93	21	1.89	44	0.08	50	0.97	50
Feather meals (n = 12)	0.12	25	3.99	33	106.42	46	5.25	60	0.12	50	0.97	50
Pork, lean meat, raw*	73.11		29.65		1952.07		73.47		0.74		54.72	
Liver, pig, raw*	28.28		168.81		3576.60		246.86		22.47		263.08	
Kidney, pig, raw*	33.89		101.86		1866.53		257.25		5.53		74.42	
Lung, pig, raw*	11.21		30.37		1851.18		79.71		1.11		3.94	
Blood, beef, raw*	0.27		6.46		27.05		0.38		1.47		3.50	
Tendon, beef, raw*	4.54		> 0.01		443.78		—		0.37		—	

* Values calculated from publication of Hegedüs et al. (1984)

The constituting elements of bones also showed a negative correlation with crude protein, offering the possibility to predict Ca, P and Mg content from crude protein values.

Crude protein — Ca: $r = -0.82$; $(P < 0.001)$
Crude protein — P: $r = -0.81$; $(P < 0.001)$
Crude protein — Mg: $r = -0.86$; $(P < 0.001)$

Vitamin B content

Principally, abattoir by-product meals are rich in protein and minerals, but their contribution to the vitamin B supply of farm animals may also be important. However, published data are relatively scarce and sometimes conflicting (Pritchard and Smith, 1957; NRC, 1969; Jeroch, 1972; Scheiner and De Ritter, 1975; Roche, 1979).

Thiamine, riboflavin, niacin, pantothenic acid, biotin and pyridoxine contents of different abattoir by-product meals determined by microbiological assays are summarized in Table IV. As was to be expected, the thiamine content of all meals was very low (0.12–0.24 μmole/kg) owing to the heat sensitivity of thiamine. Meat-and-bone meals and poultry by-product meals were a richer source of riboflavin, niacin, pantothenic acid, biotin and pyridoxine than blood meals, bone meals and feather meals.

The vitamin B levels of different abattoir by-product meal samples were significantly lower than those of the same raw materials (meat, liver, kidney, lung). On the other hand, tendon and blood were shown to be poor vitamin B sources. The lower vitamin B levels in the by-product meal samples may originate from the diluting effect of low quality raw materials (connective tissues, gut content, keratinous raw materials etc.) as well as from the heat- and oxidative deterioration of vitamins during cooking and drying. The possibility of a limited water extraction of these water-soluble vitamins cannot be excluded either.

Table V

Recommended* vitamin B levels in poultry and pig feeds

	Thiamine-HCl (μmole/kg)	Riboflavin (μmole/kg)	Niacin (μmole/kg)	Ca-pantothenate (μmole/kg)	Biotin (μmole/kg)	Pyridoxine-HCl (μmole/kg)
Broilers, starting	8.89	21.25	406.17	41.97	0.61	34.05
Broilers, growing	8.89	15.94	324.94	25.18	0.61	24.32
Piglets, starting	8.89	15.94	203.08	41.97	1.02	29.18
Pigs, growing	7.41	13.28	162.47	31.48	0.61	24.32

* Values are calculated from Vitamin Compendium, Roche, Basel, 1976, p. 34.

The pyridoxine content of all abattoir by-product meal samples was relatively low (0.97–4.86 μmole/kg). This may be the consequence of the lower heat stability of pyridoxine (Bokori and Hegedüs, 1976) than that of riboflavin, niacin and biotin.

Comparing the vitamin B potency of meat-and-bone meals and poultry by-product meals with the recommended levels in mixed feeds for poultry and pigs (Table V), their contribution to meeting the requirements of farm animals is significant. However, standard deviations of the average values were also high.

Blood meals, bone meals and feather meals proved to be negligible sources of group B vitamins.

The riboflavin ($r = 0.73$; $P < 0.01$), niacin ($r = 0.73$; $P < 0.01$) and vitamin B_6 (0.73; $P < 0.01$) contents of meat-and-bone meals showed significant positive correlations with the crude protein content. No correlation was found, however, between vitamin B and crude protein contents of poultry by-product meals. The correlations offered no possibility for prediction of vitamin B levels from raw protein content of the samples.

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THE VALUE OF CRUDE PROTEIN CONTENT AND *IN VITRO* PEPSIN DIGESTIBILITY OF ABATTOIR BY-PRODUCT MEALS IN THE PREDICTION OF THEIR AVAILABLE PROTEIN CONTENT

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Amino acid composition of meat-and-bone meals, poultry by-product meals, blood meals, bone meals and feather meals showed characteristic differences. Meat-and-bone meals and blood meals had surplus in lysine, whereas poultry by-product meals and feather meals were relatively rich in cystine. Blood meals had high levels of branched-chain amino acids as compared to isoleucine.

In vitro pepsin digestibility of meat-and-bone meals (79.9 $\pm$ 17.7%; n = 24) and blood meals (95.8 $\pm$ 4.2%; n = 11) was found to be higher than that of poultry by-product meals (65.3 $\pm$ 7.7%; n = 14) or feather meals (44.7 $\pm$ 9.2%; n = 16). Pepsin digestibility of poultry by-product meals showed a significant negative correlation with crude protein content ($r = -0.73$; $P < 0.05$; n = 14). However, *in vitro* pepsin digestibility of poultry by-product meals as well as meat-and-bone meals, blood meals and feather meals, showed insignificant correlations with NPU indices as well as with available crude protein contents of the meals.

The NPU values of meat-and-bone meals for rats (29.9 $\pm$ 11.7; n = 100) were lower than those of poultry by-product meals (52.1 $\pm$ 7.1; n = 14). The NPU values of blood meals (6.4 $\pm$ 5.6; n = 11) and feather meals (23.5 $\pm$ 10.0; n = 16), determined as sole sources of protein, were low and they did not elicit weight gain in rats.

The available crude protein content of poultry by-product meals (34.3 g/100 g $\pm$ $\pm$ 3.6; n = 14) was higher than that of meat-and-bone meals (17.0 g/100 g $\pm$ 7.3; n = 100). The available protein content of meat-and-bone meals showed a moderate positive correlation with crude protein content ($r = 0.39$; $P < 0.05$; n = 100).

The crude protein as well as *in vitro* pepsin digestibility values were inadequate for prediction of available protein content of the meals evaluated in these experiments.

Keywords: Meat-and-bone meal, poultry by-product meal, blood meal, feather meal, protein quality.

Abattoir by-product meals are primarily used as sources of protein in mixed feeds, thus most attention is paid to their amino acid content and protein quality.

It has generally been accepted that the protein quality of abattoir by-product meals can be characterized by digestibility, amino acid composition and available amino acid content of the protein, as well as by various *in vivo* indices including BV (biological value), NPU (net protein utilization), NPR (net protein ratio), etc. (Pellett and Young, 1980; Hegedüs et al., 1981). Abattoir by-product meals can be tested as sole protein sources in feeding experiments (e.g. Bunyan and Price, 1960; Lynch et al., 1970) or in mixtures

(e.g. Jackson, 1972; Johri et al., 1980; Hegedüs et al., 1983*a*, *b*, *c*). The evaluation of protein quality of by-product meals as sole protein sources is used to control different batches, whereas their testing in mixtures gives information on the supplementary potency of protein meals for cereal grains or other feed ingredients.

The great variance in the nutritive value of abattoir by-product meals is widely reported and is considered as a consequence of the differences found in their chemical composition and protein quality.

The present work was performed to study the typical differences in the protein quality of meat-and-bone meals, poultry by-product meals, blood meals and feather meals, as well as to find correlations among the parameters generally used as indicators of protein quality in order to evaluate their usefulness to predict available protein content of the meals.

Materials and methods

Origin of samples

The samples of abattoir by-product meals were taken from batches produced on an industrial scale in 9 different dry rendering plants. The raw by-products obtained from slaughterhouses were heat treated (130 °C, 30 min), which was followed by the reduction of moisture by drying (100 °C, 4 h) and lowering the fat content by pressing (Szél and Gál, 1980).

Raw materials of *meat-and-bone meals* consisted of soft offals of varying composition (confiscated organs, blood, rumen and gut of swine and cattle) approximately 60% (w/w), and of hard offals (bones, hooves, hair, feather) 40% (w/w).

The poultry *by-product meals* consisted of all slaughter offals including also feather and intestinal content.The approximate ratios of ingredients were: 10% w/w hatching residues, 50% w/w guts, heads and shanks, and 40% w/w feathers. The meals were produced in 4 different dry rendering plants according to the De Vita technology.

Bone meals were produced from industrial raw bones. *Blood meals* were produced from slaughterhouse flow-off blood in batch rendering cookers. The raw material of *feather meals* consisted of wet feathers obtained from poultry slaughterhouses. The processing of feathers was carried out without chemical treatment, only steam pressure (heating) was used.

The commercially available samples were taken from the rendering plants after their microbiological control.

Methods

Crude protein content and *in vitro* pepsin digestibility of protein meals were determined according to the Hungarian standard MSZ 6830*.

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

The determination of net protein utilization (NPU) by rat experiments was carried out as described in a previous paper (Hegedüs et al., 1983*a*). The SPF rat strain LATI CFY was used in the experiments. Available protein content of the samples was calculated by multiplying the crude protein content by NPU.

Results and discussion

Amino acid content

The amino acid data presented in Table I show characteristic differences among types of by-product meals used to supplement feeds based on cereal grains. The broad range of concentrations of individual amino acids in the

Table I

Lowest and highest levels of amino acids found in abattoir by-product meals

	Meat-and-bone meals (n = 5)	Poultry by-product meals (n = 5)	Blood meals (n = 5)	Bone meals (2) (n = 5)	Feather meals (n = 5)
Alanine (g/16 g N)	7.4—8.4	5.3—6.0	7.2—7.3	8.0—8.4	4.0—5.0
Arginine (g/16 g N)	5.2—6.8	6.7—8.4	4.6—5.2	7.1—7.4	6.1—6.8
Aspartic acid (g/16 g N)	7.3—9.6	7.0—7.8	8.5—10.5	6.8—7.0	6.4—6.6
Cystine (g/16 g N)	0.4—1.1	2.6—5.9	0.8—0.9	0.1—0.2	4.4—5.2
Glutamic acid (g/16 g N)	10.5—12.4	11.1—12.8	9.6—10.3	11.0—12.5	10.1—11.5
Glycine (g/16 g N)	8.3—16.3	8.0—10.4	5.3—5.6	16.7—17.6	8.6—9.6
Histidine (g/16 g N)	1.5—4.5	0.8—1.2	4.0—5.2	1.0—1.2	0.5—0.7
Isoleucine (g/16 g N)	1.6—3.4	4.4—5.7	1.4—3.9	1.9—2.1	4.1—4.6
Leucine (g/16 g N)	5.4—9.6	7.8—9.4	9.1—11.5	4.6—4.7	7.5—7.7
Lysine (g/16 g N)	4.8—6.9	2.6—3.4	7.1—7.4	4.0—4.4	2.0—2.3
Methionine (g/16 g N)	1.1—1.5	0.9—1.3	0.9—1.4	1.0—1.2	0.6—0.7
Ornithine (g/16 g N)	0.0—0.1	0.2—0.3	0.0—0.0	0.0—0.0	0.2—0.3
Phenylalanine (g/16 g N)	2.6—4.5	3.4—4.8	5.0—5.1	2.3—2.6	4.1—4.3
Proline (g/16 g N)	5.6—9.4	8.3—11.4	4.3—4.4	9.1—9.9	9.0—9.7
Serine (g/16 g N)	3.5—4.9	9.0—13.8	3.9—4.6	3.3—3.5	10.1—11.0
Threonine (g/16 g N)	2.7—3.3	4.3—5.1	3.4—4.2	2.4—2.5	4.2—4.3
Valine (g/16 g N)	4.3—7.3	7.5—9.2	6.4—8.8	3.2—3.7	7.4—7.8
NH_3 (g/16 g N)	0.9—1.1	1.4—1.5	1.6—1.7	0.8—0.9	1.5—1.7
Amino acid-protein (g/16 g N)[1]	76.0—78.5	80.4—100.0	77.0—78.9	74.0—75.1	81.0—82.0

1 = Protein calculated from measured amino acids, excluding tryptophan
2 = Bone meal is regarded as a source of minerals instead of amino acids

* MSZ 6830 (1981): Determination of the nutritive value of feeds (in Hungarian). Hungarian Standard, Budapest

meals is associated with the variance in raw material composition and draw attention to the uncertainty when standard figures are used from feed composition tables.

Meat-and-bone meals and *blood meals* had surplus in lysine, which is the main limiting essential amino acid in cereal grains. On the other hand, *poultry by-product meals* and *feather meals* were relatively poor in lysine but rich in cystine. This is in accordance with the findings of Jackson (1971) and other authors (e.g. Burgos et al., 1974; Baker et al., 1981).

Eggum (1970) pointed out that in spite of a great loss of cystine presumably due to lanthionine formation, *feather meals* are rich in cystine, as well as in threonine and arginine, but they are deficient in methionine, lysine, histidine, and tryptophan. The order of limiting amino acids in feather meals, as proposed by Wessels (1972), is methionine, lysine, histidine and tyrosine. These results were obtained in chickens in nitrogen retention trials combined with supplementation of feather meal with different individual amino acids.

Blood meals had high levels of branched-chain amino acids (leucine and valine) as compared to isoleucine. The gross lysine content of blood meals is relatively high, however the availability of lysine may be low due to the severe heat processing frequently used.

The imbalance among amino acids in blood meals results in suppressed growth of animals when higher levels of blood meal are used in mixed feeds (Fritzpatrick and Bayley, 1977). Higher amounts of blood among the raw materials of meat-and-bone meals may adversely affect the protein quality of the meals (Hegedüs et al., 1983*a*).

Collagen is the main protein constituent of *bone meals*. The amino acid pattern of bone meals corresponded to that of collagen, which is deficient in histidine, threonine, isoleucine, valine and tyrosine (Skurray, 1982). As collagen does not contain cystine, the cystine contents of bone meals were very low. Hydroxyproline occurs only in collagen, thus its concentration can be regarded as an indicator of the nutritive value of meat-and-bone meals (Hegedüs and Bokori, 1983). Tryptophan content was not measured.

Prediction of available protein content

The digestibility of crude protein, net protein utilization, and available protein content of different by-product meals are summarized in Table II.

In vitro digestibility of protein meals by pepsin has been proposed as an indicator of protein quality. However, it has long been recognized that pepsin digestibility shows poor correlation with *in vivo* data (Boyne et al., 1961). Thus, *in vitro* pepsin digestibility data may be used only to control the stated parameters of the batches and products.

Table II

Protein quality of abattoir by-product meals

	Meat-and-bone meals (n = 100)	Poultry by-product meals (n = 14)	Blood meals (n = 11)	Feather meals (n = 16)
Crude protein (g/100 g)	56.7±7.1	66.6±8.2	72.9±10.1	83.4±5.5
Digestibility of crude protein (%) (1)	79.9±17.7 (5)	65.3±7.7	95.8±4.2	44.7±9.2
NPU (%) (2)	29.9±11.7	52.1±7.1	6.4±5.6	23.5±10.0
Available crude protein (g/100 g) (3)	17.0±7.3	34.3±3.6	4.1±2.6	20.0±9.1
Relative weight change (%) (4)	99.7±9.6	104.8±8.9	83.3±5.5	88.9±4.3

1 = *In vitro* pepsin digestibility
2 = Net protein utilization determined on rats
3 = Crude protein multiplied by NPU/100
4 = Final body weight of rats expressed in per cent of starting weight
5 = n = 24

Table III

Correlation coefficients between crude protein and parameters used for indicating protein quality of abattoir by-product meals

	Meat-and-bone meals (n = 100)	Poultry by-product meals (n = 14)	Blood meals (n = 11)	Feather meals (n = 16)
Digestibility	0.30 (1)	−0.73*	0.12	−0.31
NPU index	0.08	−0.66*	−0.80*	0.16
Available crude protein	0.39*	0.18	−0.79*	0.29
Relative weight change	0.06	−0.83*	−0.57	0.02

* = Correlation coefficient deviates significantly from zero ($P < 0.05$)
1 = n = 24

In vitro pepsin digestibility values of meat-and-bone meals (79.9% ± 17.7; n = 24) and blood meals (95.8% ± 4.2; n = 11) were found to be higher than those of poultry by-product meals (65.3% ± 7.7; n = 14) for feather meals (44.7% ± 9.2; n = 16). The protein evaluation of bone meals was omitted, since bone meals are regarded primarily as mineral sources.

The pepsin digestibility of poultry by-product meals showed a significant negative correlation with crude protein content ($r = -0.73$; $P < 0.05$; n = 14) as seen in Table III. This could be the consequence of the varying proportion of feather in poultry by-product meals. However, *in vitro* pepsin digestibility of poultry by-product meals as well as meat-and-bone meals, blood meals and feather meals showed insignificant correlations with NPU indices, as well as with available crude protein contents of the meals.

The average *NPU value* of the large number of meat-and-bone meal samples (29.9 ± 11.7; n = 100) was lower than that of poultry by-product

meals (52.1 $\pm$ 7.1; n = 14). The variance of NPU values in poultry by-product meals was lower (CV = 13.6%) than in meat-and-bone meals (CV = 39.1%). This shows the more uniform raw material composition of poultry by-product meals.

The NPU values of blood meals (6.4 $\pm$ 5.6; n = 11) and feather meals (23.5 $\pm$ 10.0; n = 16) for rats determined as sole protein source were low. However, it is important to draw attention that these figures can be misleading, because in practice blood meals are not fed as a sole source of protein and they are more valuable ingredients, regarding their complementing potential for cereal grains, than are feather meals. Judging the protein quality of by-product meals, a clear distinction should be made between NPU value or *available protein content* and *complementing potential.*

The available protein content of poultry by-product meals (34.3 g/100 g $\pm$3.6; n = 14) was roughly twice as high as that of meat-and-bone meals (17.0 g/100 g $\pm$7.3; n = 100), indicating the superior quality of poultry slaughter by-products (gut, head, shanks). Raw materials of meat-and-bone meals are more diverse in their nature (more gut content and less meat tissues).

The figures of *relative weight change* of rats during the feeding period (Table II) show that blood meals and feather meals do not support weight gain when fed as sole sources of protein in the test diets. Similarly, more than 50 per cent of the examined meat-and-bone meal samples (54 out of 100 samples) did not support weight gain in rats. Poultry by-product meals proved to be the best in promoting weight gain owing to their higher available protein content.

Crude protein content of meat-and-bone meals showed moderate correlation (Table III) with available protein content ($r = 0.39$; $P < 0.05$: n = 100).

However, there were no significant correlations between crude protein level and available protein content of poultry by-product meals and feather meals ($r = 0.18$ and 0.29 respectively; $P > 0.05$). The negative correlation between crude protein level and available protein content of the blood meal samples may be explained by the diluting effect of other raw materials accidentally cooked together with blood during the manufacturing of blood meals, which have lowered crude protein level and improved NPU value of the final meal.

It can be concluded that crude protein levels and *in vitro* pepsin digestibility values are inadequate measures for prediction of available protein content of the abattoir by-product meal samples evaluated in these experiments.

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CHARACTERIZATION OF *PASTEURELLA HAEMOLYTICA* STRAINS ISOLATED FROM GOATS IN HUNGARY

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Thirty-five *Pasteurella haemolytica* strains were isolated in Hungary from goat carcasses sent for postmortem examination from two farms with large goat flocks. All strains belonged to biotype A and with the exception of one strain of serotype A8 they belonged to serotype A2. No untypable strains were found by the indirect haemagglutination test.

Keywords: *Pasteurella haemolytica*, goat, pneumonia.

Recently in Hungary some goat flocks comprising a few hundred animals have been established to meet the increased demand for goat meat and milk products. Breeding goats in such flocks increased the risk of various infectious diseases. Of these, pneumonic diseases are considered to have special economic importance.

The role of *Pasteurella haemolytica* in the diseases of sheep (Gilmour, 1978) and cattle (Frank and Smith, 1983; Yates, 1982) is well known. Less attention has been paid to its pathogenicity in goats.

Based on their biochemical features *P. haemolytica* strains can be designated as A and T biotypes and by means of an indirect haemagglutination (IHA) test they can be grouped into 16 serotypes (Biberstein, 1978; Fodor et al., 1987; Fraser et al., 1982; Pegram et al., 1979). Biotype A strains are responsible for pneumonia of sheep of all ages, septicaemia of lambs and mastitis of ewes, while strains of biotype T cause systemic pasteurellosis of feedlot lambs (Biberstein, 1978; Gilmour, 1978; Dyson et al., 1981).

P. haemolytica can be isolated from the nasal passages of healthy goats (Ojo, 1976) and from sheep (Biberstein et al., 1970). In the presence of predisposing factors it can cause fatal pneumonia in goats of different ages (Gourlay and Barber, 1960; Mwangota et al., 1978; Ojo, 1976; Ojo, 1977), unilateral mastitis (Bagadi and Razig, 1976), and sometimes the organism can be isolated from subclinical mastitis (Manser, 1986).

Challenge experiments in goats showed that *P. haemolytica* biotype A, when administered intraperitoneally or intravenously, killed one-day-old kids,

while live or heat-inactivated *P. haemolytica* of biotype T produced pneumonia (Gourlay and Barber, 1960; Ngatia et al., 1986).

We have little information on goat diseases caused by *P. haemolytica* in Europe so this study was carried out to determine the prevalence of the different biotypes and serotypes of *P. haemolytica* among goats in Hungary.

Materials and methods

Altogether 35 *P. haemolytica* strains were isolated from 29 carcasses of goats of different ages, mainly kids sent for postmortem examination from two farms with large flocks. Twenty-eight strains originated from pneumonic lungs, 2 were isolated from the nose and middle ear respectively and one each from brain, liver and milk. Samples were cultured on nutrient agar containing yeast extract and crystal violet–lactose–bromothymol blue agar. They were incubated at 37 °C for 24–48 h.

Strains resembling pasteurella on the basis of cultural features, colony morphology and Gram staining were examined biochemically and serologically by IHA according to Biberstein (1978).

Results

Based on biochemical characteristics all the strains examined belonged to biotype A.

With the exception of one strain of serotype A8 isolated from the lung of a nanny goat all strains were assigned to serotype A2 (97.1%). No untypable strains were found. The same serotype was isolated from both lungs and sometimes from the middle ear of the same animal.

Discussion

The results suggest that *P. haemolytica* is in part responsible for pneumonic diseases among goats in Hungary, however the number of the cases examined was relatively low.

Most reports on goat pasteurellosis are from tropical countries perhaps due to their larger goat populations (Gourlay and Barber, 1960; Mwangota et al., 1978; Ojo, 1975; Ojo, 1976; Ojo, 1977). Little work has been done in Europe on goat diseases caused by *P. haemolytica* though experience in New Zealand shows that they have importance in temperate zones as well (Midwinter et al., 1986).

Mwangota et al. (1978) isolated from healthy and diseased animals all the 12 serotypes which were known at that time and they found the same variability among them as among the strains isolated from sheep. According to their results serotype A11 was predominant; it comprised nearly 20% of their strains.

The majority of the strains isolated by Ojo (1975) in Nigeria belonged to serotype A2 as did ours, but serotypes A6, A7, A8 and A11 were also important and some of his strains were untypable.

Midwinter et al. (1986) isolated mainly strains of serotype A2 in New Zealand and the three goat strains isolated by Pegram (1974) were similarly A2.

It is of interest that with the exception of one A8 strain only *P. haemolytica* A2 strains were isolated from goats in Hungary, while in diseased sheep all *P. haemolytica* serotypes but A14 were detected (Hajtós et al., 1985). This can probably be explained by isolated keeping of goats from sheep flocks on the two farms examined.

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PREVALENCE OF *SALMONELLA* SEROTYPES IN PIGS AND EVALUATION OF A RAPID, PRESUMPTIVE TEST FOR DETECTION OF SALMONELLA IN PIG FAECES

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Two hundred faecal samples, collected from pigs at a Budapest abattoir, were examined for the presence of salmonellae. Ninety-six isolates, belonging to 7 different serotypes, were obtained. *Salmonella derby* (70.8%) was the most prevalent serotype. The other serotypes isolated were *S. typhimurium* (8.33%), *S. bredeney* (4.16%), *S. agona* (2.08%), *S. infantis* (9.22%), *S. london* (3.12%), and *S. panama* (2.08%). A modified rapid presumptive test to detect salmonellae in food and food ingredients was described by Hoben et al. (1973). This test medium was evaluated to determine its efficacy in detecting salmonellae in pig faecal samples. The test gave an efficiency of 88.67%. However, the incidence of false positive results was rather high (9.43%). The public health importance of the isolates obtained and the application of the rapid presumptive test at the farm level are discussed.

Keywords: Salmonella, pig, serotypes, rapid test.

Salmonellae are widely distributed in nature and their importance as pathogens in animals and man is well established (Ghosh, 1972). The public health importance of swine as a major reservoir of salmonellae has been well documented (Keteran et al., 1982). Studies have shown that salmonellae are generally present in a small percentage of normal healthy hogs (Edel and Kampelmacher, 1976) and that transport time, feeding, environmental contamination, and the length of time spent in the lairage may affect the salmonella isolation rate (Williams and Newell, 1968; Childers et al., 1977; Morgan et al., 1987). The present paper investigates the possibility of using a rapid, presumptive test for detection of salmonellae in pig faeces. This test was initially developed by Hargrove et al. (1971), and was modified by Hoben et al. (1973) for detection of salmonellae in food and food ingredients. The objective of this work was to evaluate the efficiency of the test in detecting salmonellae in pig faeces and to determine whether it could be recommended for use at the farm level for detecting salmonella excretors. Separation of such excretors prior to transport to the abattoir could, in all probability, greatly reduce salmonella cross contamination during transport, in the lairage and at the slaughterhouse. The study also envisages to note the prevalence of salmonellae serotypes existing in the faeces of healthy pigs obtained for slaughter at an abattoir in Budapest.

Materials and methods

Two hundred faecal samples were collected from pigs at a Budapest swine abattoir between September and November 1987. During this period the slaughterhouse was visited every week and 20 faecal samples were collected from pigs which represented a single lot from one farm. The 200 faecal samples thus represented 10 different farms.

Faeces was collected from the caecum by aseptic techniques, and about 1–2 grams of the faeces was transferred into a test tube containing 10 ml of tetrathionate (TT) broth. The samples were transferred to the laboratory within 1 h of collection and were incubated at 43 °C for 24 h. A second enrichment was made in another 10 ml of TT broth, by transferring 1 ml of the TT broth enriched for 24 h. This second enriched TT broth was incubated for another 24 h. Subcultures were made on Drigalski's agar (DA) and brilliant green–phenol red agar (BGA), at the end of the 24- and 48-h incubation of the enriched TT broths. At least three suspected salmonella colonies were picked and subjected to short biochemical tests. Colonies showing characteristic biochemical reactions and positive on examination with polyvalent "O" antisera, were then studied for their serotypes (species). The biochemical tests were performed as described by Edwards and Ewing (1972). Serotyping was done as described by Kauffman (1972).

The rapid presumptive test medium, i.e. lysine iron cystine neutral red broth (LICNR) was prepared as described by Hoben et al. (1973) except that novobiocin was not incorporated into the medium. One ml of the 24 h enriched TT broth was inoculated into 10 ml of LICNR broth, which was then incubated at 37 °C for 42 h. Tubes showing a change from red to straw colour, with a black precipitate of hydrogen sulfide, were separated. To these tubes 0.1 ml of bromothymol blue (0.3%) solution was added. Tubes showing green or blue colour on addition of the bromothymol indicator were enumerated as presumptive positive for salmonella. One loop full of LICNR medium (presumptive positive or negative for salmonella) was plated onto DA and BGA medium to isolate the salmonella organisms for confirmation by routine diagnostic procedures, in order to evaluate the efficacy of the LICNR broth.

Results

A total of 96 salmonella isolates were obtained from the 200 faecal samples analysed. These 96 isolates belonged to 7 different serotypes. Eighty-two isolates were identified as belonging to Group B, 9 isolates to Group C, 2 isolates to Group D, and 3 isolates to Group E. Only one *Salmonella* serotype was obtained from each of the positive samples. The most prevalent serotype

was *S. derby* (70.83%), followed by *S. infantis* (9.37%), *S. typhimurium* (8.33%), *S. bredeney* (4.16%), *S. london* (3.12%), *S. agona* (2.08%), and *S. panama* (2.08%) (Table I). The effect of a 24-h enrichment in TT broth, when subcultured on DA and BGA medium, showed an isolation rate of 88.54% and 90.62%, respectively, while the effect of a 48-h enrichment in TT broth, when subcultured on DA and BGA medium, was 93.75% and 95.90%, respectively.

Table I

Salmonella serotypes isolated from pig faeces

No.	Serotype	No. and % of isolates	Farm no.
1	*S. agona*	2 (2.08%)	2
2	*S. bredeney*	4 (4.16%)	5
3	*S. derby*	68 (70.83%)	1, 5, 6, 7, 9, 10
4	*S. infantis*	9 (9.27%)	2, 4
5	*S. london*	3 (3.12%)	1, 6
6	*S. panama*	2 (2.08%)	4, 7
7	*S. typhimurium*	8 (8.33%)	2, 3, 5, 7, 8

The rapid, presumptive test (LICNR) gave a total of 106 samples positive for salmonella, of which 94 (88.67%) were positive for the presence of salmonella as confirmed by plating the LICNR medium enumerated as positive onto DA and BGA medium and examined further by routine diagnostic tests. Ten samples (9.43%) gave false positive results in LICNR medium: no salmonella could be isolated from these samples. The organisms responsible for false positive results were *E. coli* and *Proteus* species (in 9 out of the 10 samples). In one sample the organism(s) could not be identified. Two samples (1.88%) gave false negative results. It was observed that an excessive growth of *Proteus* species resulted in false negative results: subsequently salmonella was isolated from these samples. The false positive tubes were identified by routine conventional isolation procedures when the samples were found positive for salmonella.

Discussion

During the ten-week period of study seven serotypes were identified. These serotypes have also been reported to have been isolated from pig faeces, pork products and pig feed in Hungary by Takács and Papp (1974), Bándy and Ákoshegyi (1976) and Papp (1981). Isolation of similar serotypes has also been reported by Harvey et al. (1977) from the faeces and lymph nodes of pigs in Great Britain and by Morgan et al. (1987) in Australia.

Kelterborn (1967) listed 47 serotypes as the most frequently isolated salmonellae all over the world. The isolates obtained in the present study belong to the most frequently isolated serotypes. *S. derby* was the predominant isolate in the present study. Similar findings were also reported by Chau et al. (1977) who isolated *S. derby* most frequently from pigs slaughtered in Hongkong. The same was found by Morgan et al. (1987) in Australia and by El-Nawawi et al. (1986) in Cairo (Egypt). Kovács and Domján (1986) reported that in 1981 through 1985 *S. typhimurium*, *S. derby* and *S. panama* were the commonest serotypes in Hungary. All these serotypes had regularly been isolated in these years from slaughtered animals, raw materials, raw processed meats, and from humans. *S. infantis* was the only serotype observed to have consistently occurred in animal feeds.

The public health importance of the serotypes isolated in the present study has been highlighted by a number of authors from other countries (Kreuscher and Sylvester, 1951; Buxton, 1957; Smith, 1964; Basu et al., 1975; Guineé and Valkenburg, 1975; Hobbs and Gilbert, 1978).

S. agona was known to have caused a significant public health problem in the Federal Republic of Germany, in the United Kingdom, Israel, and in the Netherlands during the years 1969–1970 (Clark et al., 1973). Fox (1974) reported a dramatic increase of *S. agona* in human and non-human material in the United States at the beginning of the year 1971. McCoy (1962) and Harvey and Price (1967) found that secondary enrichment procedures were valuable in the isolation of salmonellae, especially when the numbers of the competing bacteria were very high or the initial salmonella count was very low. In the present study a marginal improvement was observed in the secondary enrichment plating as compared to primary enrichment in TT broth.

The rapid presumptive test medium (LICNR) gave a presumptive positive test in 94 samples (88.67%) as compared to the conventional isolation procedures, in which 96 isolates were obtained. There are no data in the literature to support the findings observed in the present study. A high incidence of false positives (10 samples, 9.43%) was observed. Hoben et al. (1973) stated that the false positive results obtained for food samples were due to a combination of coliforms and *Proteus* species. Similar findings have been obtained in the present study. Hoben et al. (1973) incorporated novobiocin at the rate of 10 to 15 μg/ml of LICNR broth. No false positive results were obtained for the food and feed ingredients examined by them, though 94% of their samples were positive. All the presumptive tests performed were corroborated by conventional isolation procedures. In 2 samples (1.88%) false negative results were obtained. This was attributed to the excessive growth of *Proteus*, resulting in the suppression of salmonellae which probably were present in the faecal sample in a very small number. The performance of the rapid presumptive test medium in detecting salmonellae in pig faeces was found to be

good. The only drawback was the high incidence of false positive results (9.43%). It is suggested that trials should be carried out with incorporation of novobiocin in the LICNR broth to determine its effect in reducing the number of false positive reactions obtained in this study. The test is simple to perform, requires no special equipment or antisera, and could easily be adapted to testing pig faecal samples at the farm level, with minimum bacteriological laboratory facilities. If the novobiocin-containing medium proves efficient, this could prove the first step in reducing subsequent salmonella contamination occurring during transport, while being in the lairage, and also that of the slaughter equipment.

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ECONOMIC LOSSES CAUSED BY PARATUBERCULOSIS IN A DAIRY HERD: CASE REPORT

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The impact of clinical paratuberculosis in a 500 cow Jersey x Holstein dairy herd of loose housing system was followed up for three years. There was an increasing fall in annual milk production, namely 49, 474, and 1030 litres per cow, a decrease in the average age from 63.9 to 57.0 months, and of feed conversion from 60 to 39%. The effect of certain management deficiencies, existing throughout the study, is discussed.

Keywords: Paratuberculosis, cattle, economic losses, clinical picture.

Economic losses due to bovine paratuberculosis have yet to be precisely assessed. Recent estimates in the United States were US $ 1.5 billion for the national dairy industry (Merkal et al., 1975; Amstutz, 1984) and US $ 75.00 per head of infected cattle in Wisconsin (Chiodini et al., 1984). In the Netherlands, the loss was estimated at Dfl 1800–2250 per head, depending on the severity of symptoms (Benedictus et al., 1985). The reported losses vary with production and management system, immunological status of the herd and, presumably, methods of estimation.

Losses from bovine paratuberculosis were formerly assessed only in terms of the shortening of productive lifespan and the reduction of milk production of lactating cows (Buergelt and Duncan, 1978). This study attempted to monitor additional parameters in a dairy herd under a loose housing management system.

Materials and methods

Dairy herd. The herd was established in 1974. It consisted of 500 Jersey × Holstein-Friesian cows kept on deep litter, with a floor space of 10.2 m^2 per cow in the open yard and 4 m^2 per cow in the covered yard. Calving house capacity was 36 tethered cows. The pre-calving maternity house could hold 60 cows and the quarantine house 25 animals. The calf-rearing house provided facilities for single caging of the newborn calves and group rearing of the older calves.

Clinical paratuberculosis was first noted in 1981. The diagnosis was confirmed by positive bacteriological examination of the faeces and presence of pathognomonic gross and microscopic lesions post mortem.

Methods. Records on herd health and performance had precisely been kept from the foundation of the dairy farm in 1974 until its liquidation in 1985. Assessment of losses was based on reproduction, performance and health parameters. The following parameters were calculated:

1. *Total annual milk production* was determined for each year from 1974 to 1984 as percentage of the national average milk production (Hortobágyi and Sebestyén, 1981–1984). It included the colostrum fed to calves or discarded.

2. *Total annual feed consumption* was determined for each year from 1977 to 1984 and expressed in starch equivalents. Feed utilization, in terms of feed conversion to litres of milk, was expressed in absolute values and percentages of a theoretical 100% conversion.

3. *Mean age of cows* was calculated in months for each year and plotted graphically for comparison.

4. *Total losses through culling, emergency slaughter and death* were expressed as the percentage of the actual herd size in each year.

5. *Age group size* was determined for all the cows 2 to 9 years of age, in percentages of the total herd size for each year from 1981.

6. *Total losses.* All losses due to paratuberculosis and other causes were calculated for each age group in percentages relative to the herd size for each year from 1981 to 1984, and plotted graphically for comparison.

7. *Mean interval from the last calving to death or slaughter* was calculated in days for each year from 1981 to 1984. The affected cows were allocated to two categories, before or after the 200th postpartum day, in order to estimate the impact of parturition on clinical disease.

8. *Conception index and calving rate* were calculated for the years 1981 through 1984.

9. *Fetal and postpartum calf losses* were calculated from the farm records for each year between 1981 and 1984 for total loss and for abortions, stillbirths, postpartum deaths, and emergency slaughter separately. The losses were expressed in percentages of the total annual calf number, including aborted and stillborn calves.

10. *Animal density and available floor space* were calculated for each year, using both the total number of animals rotated through the unit each year from 1981 to 1984 and the average herd size for the same year. The cows were confined to a 2000 m^2 covered yard during most of the year as the 5100 m^2 open yard could only be used in dry or frosty weather.

11. *Tuberculosis prevalence rates* were determined on the basis of a positive reaction to mammalian and/or avian tuberculin tests. *Paratuberculosis prevalence rates* were determined in relation to positive complement fixation and

double radial immunodiffusion tests and a positive microscopic examination of faecal samples.

12. *Costs of treatment.* The data on cost of chemotherapeutics and fees charged were taken from the veterinary records.

13. *Incidence of paratuberculosis* was assessed by examining organs (ileum and lymph nodes) from a total of 347 cows by histological and bacteriological methods.

Results

The annual milk yield decreased by 49 litres per cow in 1981, when paratuberculosis was first diagnosed, and by 474 and 1030 litres in the following two years, respectively. There had been no significant variation in annual milk production in previous years and it was about the national average after 1978 (Fig. 1A).

Economic losses were further increased by poor feed utilization and feed conversion (Fig. 1B). In 1977 61% of the ration was converted to milk relative to the theoretical 100% conversion. The herd performance increased from 1977 to 1981 and was at the national average during 1979 and 1981. In the three years following diagnosis (1982 through 1984) only 45, 44 and 39% of the feed, respectively, was utilized for milk production. The low performance reflects the poor digestion and absorption.

The mean age of the cows decreased from 63.9 to 57.0 months after paratuberculosis had become established (Table IV). Although the causes of culling, death and emergency slaughter varied, the total losses rose from 23.3% in 1979 to 28.5% in 1981. In the subsequent three years, losses from paratuberculosis alone amounted to 8.5, 10.5 and 15.6%, respectively.

The age group distribution of the producing herd (Table I) indicated a decreasing productive lifespan from 1981 onward. Most cows lost because of paratuberculosis were five years old, while those lost because of other causes were usually in the range of three to six years (Fig. 3). During the period 1981 through 1984 cow losses from death or emergency slaughter rose from 3.9 to 13.0% in the immediate postpartum period, from 2.1 to 9.3% for the period up to postpartum day 200, and from 1.8 to 3.7% for the subsequent period. Within 200 days of calving, the mean time of death or emergency slaughter was the 100th day in 1981 and the 81st day in 1984. Overall, the average interval between the last calving and death decreased from 382 days in 1981 to 314 days in 1984 (Table II).

The pregnancy and calving rates were at the usual level during the years of the study (Table III).

Total calf losses increased from 9 to 16% during the five-year period between 1980 and 1984 (Figs 2 and 4) and were over the national average

Table I

Percentage distribution of cows by age between 1981 and 1984

Year	Age of cows in years							
	2	3	4	5	6	7	8	9 and more
1981	15.4	21.3	18.0	15.0	8.6	5.4	3.8	12.5
1982	17.8	20.5	20.3	14.0	10.6	5.7	3.3	7.6
1983	22.3	19.0	22.5	14.3	7.9	5.9	3.0	5.1
1984	23.4	20.7	19.2	13.0	10.0	5.6	3.8	4.3

Table II

Data of postpartal emergency slaughters and deaths

Period	1981		1982		1983		1984	
	Cows lost	Average day	Cows lost	Average day	Cows lost	Average day	Cows lost	Average day
Before postpartum day 200	10	100	14	77	16	84	38	81
Beyond postpartum day 200	8	382	6	311	8	364	15	314
Total	18	148	20	147	24	171	53	147

Table III

Reproductive efficiency of the herd between 1981 and 1984

Year	Conception index (inseminations per conception)	Calving rate (%)
1981	1.9	88
1982	2.0	86
1983	2.2	84
1984	2.1	85

Table IV

Size of the dairy herd between 1974 and 1984

Year	Herd size (cows)			Total no. of cows rotated in the herd	Mean age (months)
	Mean	Minimum	Maximum		
1974	366	342	375	391	29.1
1975	518	419	498	617	40.5
1976	536	427	617	647	49.8
1977	549	444	619	655	54.9
1978	570	449	642	692	58.5
1979	595	442	702	749	61.9
1980	626	426	756	827	60.1
1981	462	357	534	773	63.9
1982	394	384	478	671	60.6
1983	426	406	497	731	58.0
1984	467	405	574	669	57.0

during the last three years. The increase was the steepest from 1981 to 1982. While calf losses due to death increased from 0.6 to 7.3%, the percentage of losses due to abortion and stillbirth did not change during the study. The losses among the calves of the 53 cows that died or were slaughtered before postpartum day 200 only amounted to 12% (6 calves) which is less than the herd level. Regular tuberculin testing, performed annually from 1974 to 1980 and twice a year thereafter, as well as laboratory examinations of dead and slaughtered animals demonstrated that the herd was free of tuberculosis during the study. The proportion of mammalian tuberculin reactors varied between 0 and 1.3% (Fig. 2). Cows with a suspect or positive reaction were culled immediately. For this reason, annual culling rate was always less than 1%.

Paratuberculosis losses due to culling and emergency slaughter amounted to 8.5% per annum between 1981 and 1983, and rose to 15.6% in 1984. A comprehensive complement fixation test and gel precipitation screening of the herd in 1983 identified 6.3% as positive reactors; 4.8% were identified by faecal examinations. These proportions rose to 15 and 12% respectively, in 1984. The initial avian tuberculin testing in 1982 indicated no herd infection with *M. paratuberculosis*. But in 1983 and 1985 10.5 and 29.7% of the cows, respectively, reacted positively to this test (Fig. 2). The serological and faecal examinations were positive only when the animals were clinically ill.

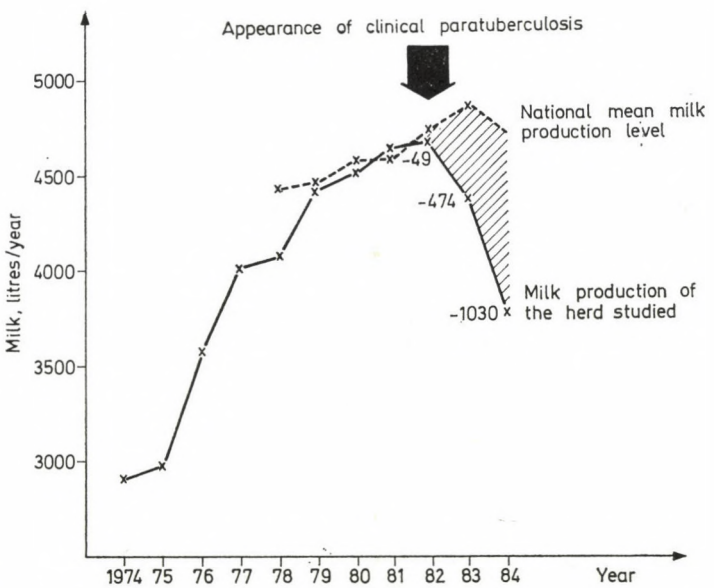

Fig. 1A. Annual milk production (relative to the national average)

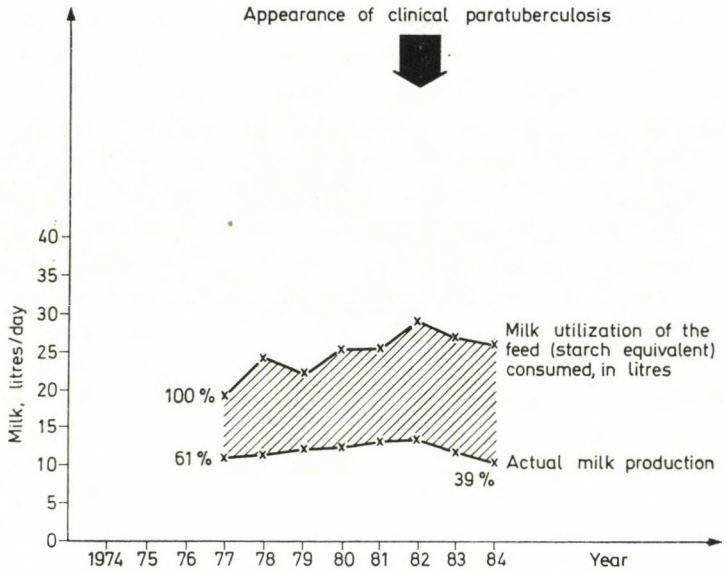

Fig. 1B. **Feed consumption in terms of starch equivalent and milk production (litres)**

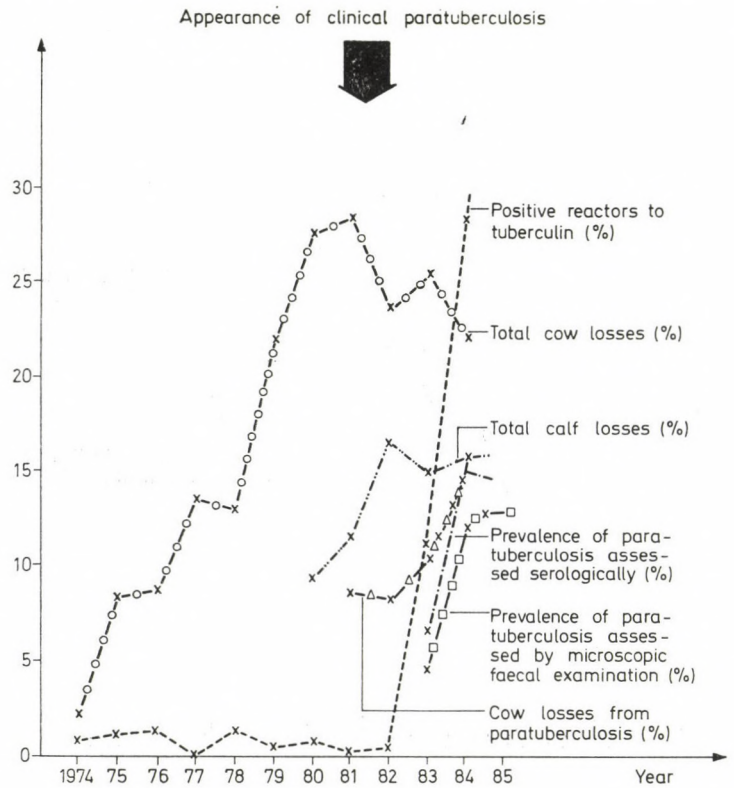

Fig. 2. **Results of paratuberculosis screening between 1974 and 1985**

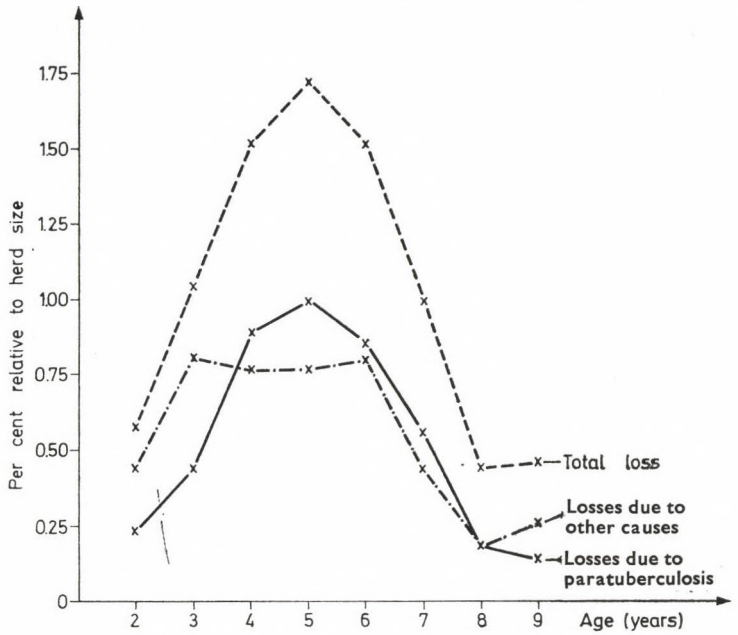

Fig. 3. Percentual cow losses according to age group between 1981 and 1984

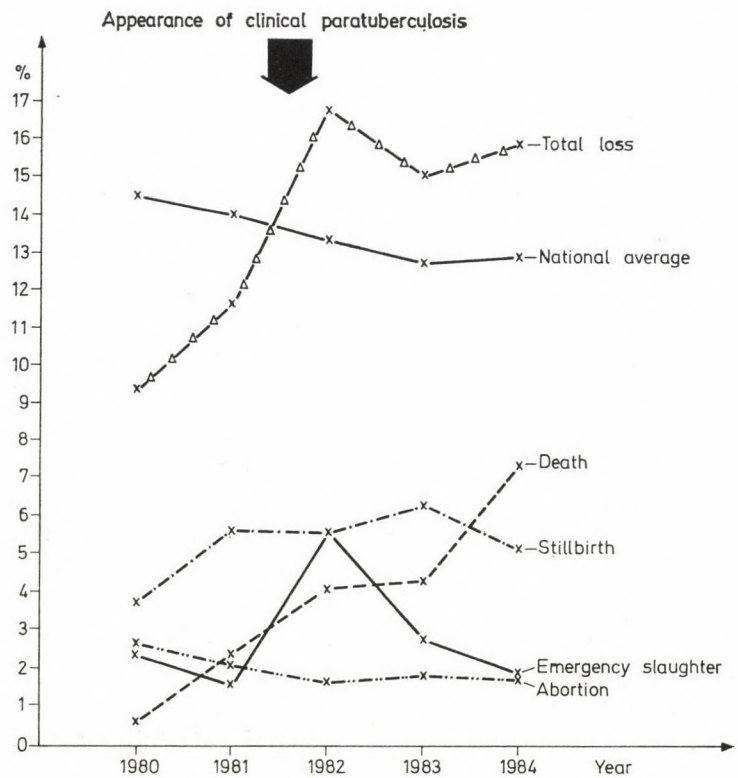

Fig. 4. Percentual calf losses between 1980 and 1984

The cost was increased by the costs of these diagnostic tests and those of the unsuccessful treatment of 80 affected cows. These costs were equivalent to the value of 80 litres of milk per cow.

Animal density (available floor space per cow) showed large variation during the study. Therefore, the covered yard was frequently overcrowded. Between 1981 and 1984 the average available space was 5.6, 5.2, 4.6 and 4.2 m^2, respectively, and when the cow numbers were highest the space was 3.7, 4.2, 4.02 and 4.5 m^2, respectively (Table IV).

Characteristic histopathological changes were observed in the ileum and the associated mesenteric lymph nodes of 91 cows out of 347 animals slaughtered. Mycobactin-dependent *M. paratuberculosis* was isolated from 23 cows of the 36 animals randomly selected from the 91 cows.

Discussion

As far as possible, the losses occurring in the herd were segregated by aetiology to obtain more precise information on the paratuberculosis situation. The involvement of some unidentifiable factors cannot be excluded. However, apart from the 12 to 15% mastitis rate, there were no important infectious diseases in the herd, nor did the quality of feed change appreciably during the study.

It appears that large herd size and drawbacks of loose housing such as overcrowding and poor hygiene might promote the clinical manifestation of this disease. In the overcrowded loose house the initial signs may pass unnoticed. The later increase in clinical problems was associated with considerable economic losses, confronting the farm manager and the animal health service with almost unsolvable problems.

Overcrowding ultimately leads to a shorter lactation, udder diseases, reduction in resting and feeding space, disturbances of the normal diurnal rhythm of feeding and milking, accumulation of sludge and manure in the yard, and to a general deterioration of management and hygiene (Amstutz, 1984; Chiodini et al., 1984). While we were able to assess the disadvantages of overcrowding, the economic evaluation of the impact of overcrowding and other aspects of poor management practices such as underutilized housing and machinery was ignored. Our observations failed to demonstrate any effect of this disease on reproduction as described by other authors (Gilmour, 1985; Merkal, 1984; Chiodini et al., 1984). The pregnancy rates of the herd affected by paratuberculosis did not differ from the national average. It is possible that culling and slaughter could have ameliorated such effects.

This study could not assess what percentage of calf losses was due directly or indirectly to maternal paratuberculosis. Mortality rates for calves

of severely affected cows were lower than the herd average. There was no evidence for, or against, specific depressive effects of maternal disease on calf viability.

Because of the ever changing economic conditions of cattle production, we believe that disease losses should be assessed in health and reproduction parameters rather than in monetary terms. The monitoring system described in this report appears to be suitable for assessing losses due to paratuberculosis and for planning preventive measures not only in dairy herds but also in beef and dual purpose herds under different management conditions.

Acknowledgement

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BONE FORMATION AND BODY COMPOSITION OF EUROPEAN ELK: AN ONTOGENETIC MODEL

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Metapodial and femur measurements were gathered on 8 female and 12 male western European elk *(Alces alces* [L. 1758] *alces)* skeletons in four osteological collections in Scandinavia. Additional variables such as carcass and bone weight as well as metacarpal measurements recorded on 43 females and 47 males hunted in Central Sweden were completed by metatarsal measurements available for 28 female and 29 male individuals of the same sample. These bones were divided into two gross age groups on the basis of epiphyseal fusion. Sex-dependent development of the metapodial bones was studied in terms of longitudinal growth and in relation to carcass weight and a number of important carcass characteristics. While metacarpals in both sexes grow at a slower rate relative to carcass weight than do metatarsals, skeletal development is more intensive in young females resulting in mature forms at a smaller absolute size. Analysis of the two sets of data was completed with parameters of dissection statistics from the literature and integrated into an ontogenetic model. A 100 to 140 kg carcass weight interval was found critical both in terms of weaning and the onset of sexual maturation.

Keywords: Ontogeny, skeletal growth, carcass composition, European elk, factor, analysis.

European elk is hunted throughout Sweden and the annual harvest of this large game has exceeded 100,000 individuals since 1979 (Hawley, 1985) thus contributing a significant portion to meat supplies in that country (Horn, 1982). Such exploitation of the population, especially in areas with relatively dense human settlement, offers a compromise between environmental and short term economic considerations, since kill-off patterns have recently shifted toward a greater proportion of younger individuals (Lundmark, 1983). Yearlings have provided a major part (40%) of harvest since the intensification of elk hunting during the late seventies (Stålfelt, 1983; Fig. 1). A better understanding of growth of elk is becoming increasingly important. On the level of basic research, evaluation of the postnatal ontogeny of elk may give insight into phenomena which have long been modified by increased variability in domestic ruminants due to selective breeding (Wilson et al., 1982).

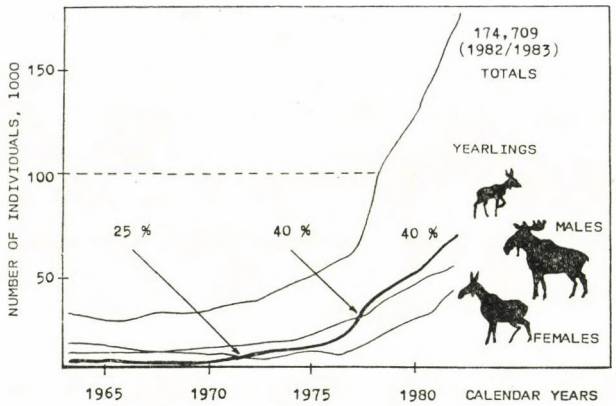

Fig. 1. The number of elk killed during the last two decades in Sweden. The contribution of young individuals to the gross increase of harvest is indicated by a heavy line

Hypothesis and test implications

Preliminary studies of the bone measurements showed sexual dimorphism in the growth of carcass weight relative to metatarsal median length. The functions shown in Fig. 2 may be described by the following allometric (Huxley, 1932) equations:

$$\text{Females: } \log CW = 3.938 \log ML - 7.811$$
$$\text{Males: } \log CW = 4.359 \log ML - 8.834$$

where CW stands for carcass weight (kg) and
ML stands for the median length of metatarsal bone (mm)

Both of these equations are supported by high linear correlations ($P \leq 0.001$). Difference shown by the allometric coefficients (italics) is obviously only a symptom of age dependent global changes in the animal's body. According to the Hammondian principles of differential growth (Hammond, 1952), these are manifested both in the skeletal features and in a variety of later maturing tissues of which muscle ("lean meat") and fat are the most important from an economic point of view. In the present paper a more concrete description of these changes was attempted on the basis of parameters obtained from a variety of data sets.

It is noteworthy that all major changes in the body conformation of elk occur at around 75 to 125 kg carcass weight. This weight for males and females is equal at the lower end of the interval which corresponds to 4.5 months of age (Skuncke, 1949). Dissection data in the literature show that decreasing bone content of the carcass also starts displaying sexual dimorphism at this point. In addition, this age may also be associated with weaning (Van

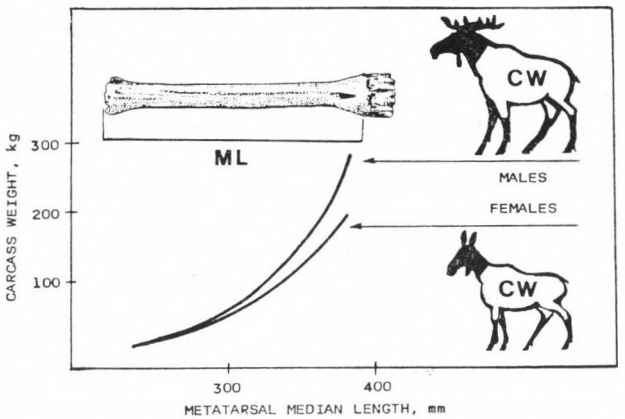

Fig. 2. Sexual dimorphism of carcass weight (CW) as predicted from the median length of metatarsals (ML). This allometric relationship served as a starting point to hypotheses tested by the ontogenetic model

den Brink, 1957). The natural age of weaning may be correlated with a number of morphological traits in cattle as well (Bartosiewicz, 1980).

Metacarpal epiphyseal fusion was observed between 1 and 2 years of age in the Hällefors population (Iregren, 1985), before full maturation. Total ossification of the distal epiphyseal plates in these bones may be expected by around the third year of the animal's life (Habermehl, 1985).

Since high correlations between skeletal measurements and carcass weight provided sex-dependent trends the following research hypotheses were formulated:

(1) The sources of skeletal variability should be separable in terms of a few background variables which include sexual dimorphism.
(2) Statistically significant stochastic relationships should justify the integrated evaluation of various data from diverse sources.
(3) The results should unambiguously correspond to other developmental phenomena discussed in this chapter.

Should all these hypotheses prove acceptable during analyses, diverse parameters could be interpreted within the framework of a general model. Such a model would outline gross tendencies of sex dependent growth in a number of body dimensions including skeletal measurements and body composition data.

Due to the non-experimental nature of the sample, test results were rejected unless significant on the $P \leq 0.001$ level of probability. In the factor analyses used in the discussion of results a $\lambda \geq 1$ threshold was set for latent roots. The significance of factor loadings was interpreted following Sváb (1979). In the appraisal of coefficients of correlation, as well as factor loadings definitions by Guilford (1956) served as guidelines.

Materials and methods

Osteometric data were gathered on complete elk skeletons (8 females and 12 males) in the three Swedish and a Norwegian osteological collections. The composition of the sample as well as the list of institutions are shown in Table I. In the State Historical Museum of Stockholm an additional set of metacarpal and metatarsal measurements were taken from the 1971 annual harvest of the Hällefors Sporting Club. They are shown in Tables II and III. Associated metacarpal measurements and theoretical densities of those bones were published by Bartosiewicz (1986*a*). A detailed description of the origin of these data was given by Iregren (1975).

Most bone measurements were taken as defined by Duerst (1926) and von den Driesch (1976). In addition to greatest metapodial lengths, however, median length (ML) was also introduced. It is measured between the most proximal end of the bone and the fusion line of the distal epiphysis. Thus, it

Table I

Measurements taken on complete skeletons (right side)

	Inv. no.	Femur GL	Metatarsus							
			GL	PB	SB	DB	PD	SD	DD	ML
Females										
OFL		478.2	387.2	50.2	31.4	64.5	52.6	32.1	41.2	358.5
ZMO	6465	326.3		45.5	21.6	57.4	47.2	26.2	40.7	290.9
ZMO	16668	428.6	375.2	51.8	29.1	64.8	54.1	31.2	45.7	348.1
OFL	KO–242	458.2	394.9	52.5	34.8	63.1	51.6	30.8	41.1	368.2
NHMG	8792	449.7	388.0	51.7	32.5	66.2	54.2	32.1	44.1	359.5
NHMG	9957	478.8	407.0	52.4	31.9	65.7	54.8	31.1	43.2	372.5
NHMG	11857	299.9	310.1	41.2	20.7	51.8	41.6	23.6	36.1	277.0
NHMG	10853	343.5	332.9	43.2	23.5	61.7	48.5	23.1	44.1	293.5
Males										
OFL	KO–240	447.2	404.2	50.2	33.1	62.8	51.7	32.8	39.5	370.2
LUND	5865	457.5	408.2	57.1	36.9	68.7	60.6	33.6	44.2	380.8
OFL		487.2	407.8	54.8	33.2	70.2	56.3	34.1	47.1	375.1
ZMO	584	498.4	406.9	56.5	34.1	69.3	58.3	34.6	47.1	384.2
NHMG	2574	226.1	262.1	37.2	16.6	44.9	37.1	15.6	32.1	234.3
NHMG	8793	476.8	386.3	48.9	32.9	63.4	53.1	29.9	40.3	360.7
NHMG	4645	457.8	403.9	49.2	31.0	64.2	55.5	32.9	41.2	376.0
NHMG	10636	364.5	361.0	51.8	28.9	69.5	52.7	31.2	45.8	334.2
NHMG	11518	445.2	386.5	52.8	34.9	67.8	54.1	33.4	46.2	357.3
NHMG	12062	462.2	400.0	53.9	34.8	67.2	54.1	31.2	43.6	370.0
NHMG	10588	433.6	401.8	50.8	30.5	61.1	54.0	29.8	41.2	377.7
NHMG	3407	498.2	412.1	56.9	34.0	69.2	57.1	33.5	45.0	389.2

Abbreviations: GL = greatest length; ML = median length; PB = proximal breadth; SB = smallest depth; DB = distal breadth; PD = proximal depth; DD = distal depth; NHMG = Museum of Natural History, Göteborg; OFL = Osteological Research Laboratory, Solna; ZMO = Zoological Museum, Oslo; LUND = Zoological Museum, Lund; Inv. no. = inventory number. All measurements in mm

became possible to include a great number of immature individuals in the ontogenetic series in spite of the fact that their distal epiphyseal plates had not yet ossified at the time of the kill. Greatest length and median length together with the transversal measurements listed in the tables were alternatively used in estimating the theoretical density of metapodials on the basis of air dried bone weight (Bartosiewicz, 1986*a*). The robustness of metapodials was expressed in the proportion of smallest breadth of the diaphysis to median length. The proportion of femur to greatest metatarsal length was used as an index (I) of the advancement of growth (Klein, 1964).

Using carcass weight as a common denominator, parameters from other investigations were included into the ontogenetic model developed in this study (Hansson and Malmfors, 1978; Hawley et al., 1983). Body composition data of animals from largely the same broader geographical region (Central Sweden) shot during the regular fall hunting season were deemed compatible with the osteological material from the Hällefors area.

Table II

Metatarsal measurements and carcass weights (CW) of female elk killed by the Hällefors Sporting Club (State Historical Museum, Stockholm). For abbreviations see Table I

Inv. no.	GL	PB	SB	DB	PD	SD	DD	ML	CW kg	SIDE
5	376.9	46.9	30.0	59.9	50.8	28.3	38.1	346.9	488	Sinister
17	385.7	47.4	30.8	59.1	52.3	28.9	41.1	353.2	493	Sinister
19	397.7	49.9	29.9	59.9	51.0	30.0	40.2	356.8	525	Dexter
21		48.2	32.1	63.2	49.9	33.1	43.1	329.6	402	Sinister
24	399.6	57.1	37.8	68.2	57.2	31.6	35.2	370.8	733	Dexter
29		41.9	21.9	52.0	44.1	25.4	40.2	271.3	220	Sinister
46	327.0	48.6	26.9	64.2	50.4	28.2	44.1	297.5	341	Dexter
50		41.1	23.2	59.2	47.0	26.1	41.3	282.9	225	Dexter
51		45.4	24.3	58.4	47.1	27.6	42.2	278.2	233	Sinister
55		46.1	24.1	59.7	46.1	26.3	43.1	273.2	235	Dexter
59	361.0	51.6	30.6	61.9	53.1	29.1	40.9	333.9	463	Dexter
60	384.1	48.4	32.1	64.2	52.9	31.4	43.2	343.2	550	Dexter
64		48.2	29.9	58.3	50.0	29.9	43.8	328.2	417	Sinister
66	385.8	49.7	30.3	62.3	53.5	30.0	42.9	356.8	520	Dexter
68	389.8	49.4	30.1	62.1	53.2	29.5	42.9	362.2	516	Dexter
74	379.6	50.9	30.8	60.2	52.9	31.1	42.5	346.0	529	Dexter
82	351.2	42.5	27.1	57.5	46.3	28.3	39.4	327.1	402	Dexter
85		51.2	26.2	66.3	50.2	29.9	47.9	319.0	279	Dexter
86		46.1	26.7	61.1	49.5	28.9	45.1	282.0	290	Sinister
94	389.2	51.9	31.1	65.2	55.4	33.2	46.4	357.9	539	Sinister
95		46.8	26.0	61.9	46.1	28.1	42.1	274.1	234	Sinister
98		46.0	22.5	56.9	46.9	26.5	44.0	278.1	236	Dexter
99		46.9	25.8	63.2	48.2	27.7	44.1	279.8	243	Dexter
103		42.9	23.0	57.2	44.5	26.2	41.5	275.6	234	Sinisser
116		46.9	25.9	63.5	47.9	28.7	42.2	279.5	242	Dexter
118	374.9	47.9	31.1	60.4	53.2	30.8	40.8	348.2	460	Sinister
121		50.9	28.2	65.4	51.5	30.7	40.9	289.6	296	Sinister
123	372.1	49.1	34.6	60.9	53.0	31.2	40.2	344.4	578	Sinister
124		43.4	23.5	53.0	45.6	26.1	39.7	276.5	218	Sinister

Cold carcass weights served as independent variables in regression analyses aimed at estimating missing values in all the data sets under discussion in this study (Frane, 1976, 1981). Using this method, carcass traits for 20 male and 16 female individuals were simulated for the purposes of the ontogenetic model. As opposed to absolute age, the size criterion minimizes environmental bias in the results (Fábián, 1969; Price and White, 1987). This is particularly important when a sample of wild animals is studied. The absolute size of elk is known to be variable even within the same region and is strongly influenced by nutrition and even weather conditions in addition to inherited factors (Stålfelt, 1983). Carcass weight is also to be preferred to total body weight as a trait less influenced by these oscillating individual conditions (viscera, intestinal fat deposits etc.; Pálsson and Verges, 1952).

In addition to univariate statistics, relationships between the variables were evaluated by factor analyses. This method investigates the degree to which clusters of intercorrelated variables may represent fewer, underlying, more basic hypothetical variables (Williams, 1979). Calculations were carried

Table III

Metatarsal measurements and carcass weights (CW) of male elk killed by the Hällefors Sporting Club (State Historical Museum, Stockholm). For abbreviations see Table I

Inv. no.	GL	PB	SB	DB	PD	SD	DD	ML	CW kg	SIDE
1	387.2	51.9	30.9	62.1	54.8	31.6	42.3	357.2	586	Dexter
3		49.9	30.5	69.1	51.8	32.4	47.8	329.3	456	Sinister
5	368.9	52.4	32.3	63.2	57.1	29.9	42.9	339.0	579	Sinister
9		51.2	28.9	66.8	53.1	31.2	45.0	329.2	444	Sinister
16		50.4	28.3	67.1	52.0	29.5	46.1	286.1	295	Sinister
18	379.1	46.7	33.7	60.1	49.3	31.0	38.9	349.0	501	Dexter
20	380.9	44.2	28.1	62.5	48.5	30.5	43.9	341.2	454	Sinister
22		48.1	29.6	62.9	48.9	29.4	44.1	324.2	400	Sinister
28	384.8	51.1	32.8	55.4	56.1	32.1	43.8	369.1	641	Sinister
47	377.4	49.1	28.8	60.8	49.1	28.3	40.2	350.0	514	Dexter
52	396.2	53.1	32.6	67.9	54.7	34.1	43.9	369.2	557	Sinister
56		48.5	25.1	63.1	48.1	27.5	45.5	284.1	279	Dexter
58		50.7	25.8	63.2	50.8	28.9	47.1	290.9	279	Sinister
61		48.9	25.0	60.0	48.1	28.1	44.5	282.1	257	Sinister
62	376.4	52.0	33.9	66.9	59.9	33.1	42.5	346.0	627	Dexter
67		45.9	24.3	57.4	47.1	27.1	44.1	283.2	226	Dexter
83		47.9	26.6	62.5	51.1	31.0	45.9	295.0	262	Dexter
84		50.1	27.3	66.5	53.7	30.1	47.9	294.2	307	Sinister
89		45.3	23.9	57.5	46.9	26.2	43.0	285.2	222	Dexter
90		50.0	29.3	72.4	52.4	34.9	50.1	333.9	439	Dexter
SM1		52.0	31.4	69.2	52.9	32.9	45.7	342.8	515	Sinister
100		47.2	25.5	64.6	48.3	29.2	46.0	307.2	329	Dexter
101		46.5	25.4	61.5	47.9	28.0	44.0	274.2	211	Sinister
105		46.9	23.9	59.5	46.2	26.5	43.2	279.5	222	Sinister
106	389.4	51.5	30.0	69.1	51.7	32.4	46.0	335.2	573	Sinister
111	386.0	51.0	33.6	62.5	54.9	35.9	40.7	354.1	627	Sinister
119	365.0	51.6	30.7	66.8	56.2	33.1	44.9	340.2	555	Sinister
126		49.3	25.1	60.1	49.5	28.5	45.8	281.2	278	Sinister
148		46.0	24.4	59.1	48.7	28.0	43.2	283.1	236	Sinister

out using a Varimax rotation in order to obtain better defined factor loading patterns and more balanced latent roots (Frane et al., 1981). Of the variables, sex and the state of distal epiphyseal fusion of metapodials were included as dichotomized ("dummy") variables (females and calves with separated epiphyses: 0, males and individuals with at least partially fused epiphyses: 1). This was made possible by the symmetric distribution patterns of the data set in terms of these variables (Sváb, 1979) which is shown by the relevant mean values ($\pm \approx 0.5$) in Tables IV and VI.

Results

As is shown by the correlations in Table IV, with the exception of sex most variables of the literature analysed in relation with the osteological data from Hällefors are significantly correlated with each other. Negative correlations occur only in relations to the proportion of front to hind metapodials first presented in this article (MCML/MTML). Obviously, relative growth of metacarpals is, in general, less intensive since they support the greater part of live weight which is not as evenly distributed as carcass fore- and hindquarter weight. Thus heavier front parts of the body (head, and especially antlers in aging males) result in excess pressure exerted on the articular surfaces. This is known to intensify the ossification process of epiphyseal plates which ultimately results in the cessation of longitudinal bone growth (Hert, 1964). According to Bergström and Wijngaarden-Bakker (1983), the density of metatarsals increases slightly more intensively relative to total bone weight in carcass side in young cattle (growing bulls), than the density of metacarpals.

Table IV

Univariate statistics and coefficients of correlation of the osteological data set. Italics: $P \leq 0.001$

	Sex	CW kg	TD g/cm³	MCSB / MCML	MTSB / MTML	MCML / MTML	Fusion
$\bar{x}$	0.520	136.341	0.382	0.107	0.088	0.874	0.545
S.E.	0.045	5.739	0.008	0.001	0.000	0.002	0.049
c.v.	0.964	0.467	0.223	0.073	0.058	0.019	0.917
Sex	1.000						
CW	0.200	1.000					
TD	0.085	*0.813*	1.000				
MCML/MCSB	0.221	*0.470*	*0.462*	1.000			
MTML/MTSB	0.229	*0.339*	0.267	*0.728*	1.000		
MCML/MTML	0.173	*−0.479*	*−0.336*	−0.286	0.023	1.000	
Fusion	0.037	*0.796*	*0.664*	*0.350*	0.229	*−0.453*	1.000

Abbreviations: TD = theoretical density; MCSB = metacarpal smallest breadth; MTSB = metatarsal smallest breadth; MCML = metacarpal median length; MTML = metatarsal median length; $\bar{x}$ = mean value; S.E. = standard error; c.v. = coefficient of variation

Table V

Rotated factor matrix of the osteological data set rearranged in the decreasing order of loadings on the first factor. For abbreviations see Table IV. Italics: $P \leq 0.001$

		FACTOR 1 Age	FACTOR 2 Sex	Communalities	
				h	%
CW		*0.881*	0.294	0.863	12.328
Fusion		*0.862*	0.114	0.756	10.800
TD		*0.812*	0.254	0.724	10.343
MCML/MTML		*−0.728*	0.233	0.584	8.343
MTSB/MTML		0.167	*0.847*	0.745	10.643
MCSB/MCML		0.426	0.437	0.725	10.357
Sex		−0.111	*0.640*	0.421	6.014
Latent roots	λ	2.930	1.889	4.818	
	%	41.851	26.977		68.828

The factor analysis, carried out using original osteometrical data gathered for this study, resulted in two synthetic, "background" variables. These may be identified as age and sex (Table V). The first factor is bipolar (Harman, 1967) and encompasses the majority of total variance. It may well be recognized on the basis of carcass weight's definitive role and the already discussed decrease of the MCML/MTML proportion. Almost one third of the total variance is represented by sex related components of the studied characteristics. Of these, the relative robustness of metatarsals in males is shown by the positive loading of the MTSB/MTML ratio. When theoretical bone densities are plotted against carcass weight, trends outlined by the obtained linear regression equations (Bartosiewicz, unpublished) show some difference between sexes. This is, however, difficult to evaluate due to the heteroscedasticity of data. Actually, as opposed to the relative robustness of metatarsals the theoretical density of these bones seems to be directly related to the carcass weight (and thus age), more or less independently of the animal's sex (Table V). Larger males, naturally, develop denser bones although rate of increase is not strongly manifested in the loading on the second factor associated with sex.

Significant correlations are even more frequent when carcass characteristics from the literature are included (Table VI), although carcass weight itself was not directly taken into consideration in this calculation. Sex, on the other hand, is an exception in this case as well. When the correlation matrix is synthetized by a factor analysis, again two factors are obtained. Due to the high number of interrelated variables, however, age includes a much greater proportion of total variance (Table VII). (The inclusion of carcass weight would have worsened this effect due to its high correlations). Meat/bone ratio, increasing metatarsal lengths and decreasing bone percentage as

Table VI

Univariate statistics and coefficients of correlation of the body composition data used in the ontogenetic model. Italics: $P \leq 0.001$

	Sex	Fusion	MTML mm	Bone %	Meat %	Fat %	ADG g	I	Fore/Hind	Meat/Bone
$\bar{x}$	0.555	0.611	335.2	19.0	76.7	4.2	0.232	1.285	0.969	4.143
S.E.	0.085	0.084	7.375	0.566	0.402	0.197	0.019	0.613	0.132	0.116
c.v.	0.907	0.809	0.130	0.176	0.031	0.275	0.477	0.283	0.081	0.166
Sex	1.000									
Fusion	0.204	1.000								
MTML	0.150	*0.787*	1.000							
Bone	0.156	*−0.644*	*−0.874*	1.000						
Meat	−0.075	*0.674*	*0.909*	*−0.974*	1.000					
Fat .	−0.296	0.473	*0.656*	*−0.885*	*0.755*	1.000				
ADG	−0.093	*−0.748*	*−0.813*	*0.715*	*−0.731*	*−0.583*	1.000			
I	0.075	*0.788*	*0.983*	*−0.881*	*0.923*	*0.648*	*−0.800*	1.000		
Fore/Hind	0.391	*0.679*	*0.638*	*−0.566*	*0.642*	0.317	*−0.555*	*0.641*	1.000	
Meat/Bone	−0.155	*0.674*	*0.904*	*−0.975*	*0.966*	*0.828*	*−0.753*	*0.923*	*0.576*	1.000

Abbreviations: ADG = average daily gain; I = femur/metatarsus length ratio, Fore/Hind = forequarter/hindquarter ratio; Meat/Bone = meat/bone ratio

Table VII

Rotated factor loading matrix of the body composition data set rearranged in the decreasing order of loadings on the first factor. For abbreviations see Table VI. Italics: $P \leq 0.001$

	FACTOR 1 Age	FACTOR 2 Sex	Communalities	
			h	%
Meat/Bone	*0.984*	−0.047	0.971	9.710
Bone	*−0.979*	0.083	0.965	9.650
Meat	*0.966*	0.042	0.935	9.350
I	*0.938*	0.223	0.930	9.300
MTML	*0.927*	0.269	0.932	9.320
Fat	*0.840*	−0.325	0.811	8.110
ADG	*−0.809*	−0.261	0.723	7.230
Fusion	*0.748*	0.439	0.751	7.510
Sex	−0.129	*0.911*	0.846	8.460
Fore/Hind	0.604	0.609	0.737	7.370
Latent roots λ	6.901	1.700	8.601	
%	69.010	17.000		86.010

well as carcass average daily gain (ADG) through life are clearly age dependent. Sexual dimorphism, which occurs in carcass weight, is somewhat apparent in the length ratio of femur/metatarsus proposed by Klein (1964) as a sensitive index of the completion of growth. The length of femora increases relative to the early fusing metapodials and this tendency is stronger in bulls (Bartosiewicz, 1987*a*). The proportion of forequarter to hindquarter weight is visibly higher in bulls although significant differences between the mean values of these parts were found only in male calves whose forequarters were actually less developed (Hawley et al., 1983). Increased growth of the neck and shoulder musculature is stimulated during sexual maturation by the increasing level of testosterone (Gerrard et al., 1987). This sexual dimorphism is even more emphasized during the rutting season in cervids due to the increased vascular supply to these muscles of the carcass in males (Field et al., 1985). At the same time, factor loadings show that the fore/hindquarter proportion is the most equally correlated with the factors obtained by this calculation: it is influenced by both age and sex.

Discussion

On the level of variables, clustering of both original bone measurements and body composition traits (univariate statistics of which were published in the literature) in two factors is particularly interesting. This integrated, multivariate approach had its forerunners. Skeletal development and fattening

results in several breeds of cattle formed three factors (Bartosiewicz, 1986*b*). In the case of that large ruminant species too, age was predominant, followed by sex. A third factor, however, interpreted as intraspecific variability, could also be extracted. This was not possible in the case of elk using the $\lambda \geq 1$ critical value for latent roots. The two factors (age and sex) were sufficient to describe most of the common variance represented by the variables as is shown by the high percentage of latent roots in Tables V and VII. This phenomenon is, to some extent, influenced by the choice of variables, but it is also due to the greater homogeneity of these data sets resulting from the better canalization of growth in wild animals under normal circumstances (Reeve and Waddington, 1952).

Results of the original analysis suggest that sexual dimorphism of the studied skeletal characteristics is chiefly related to sex-dependent differences in carcass weight. Thus, one may assume that the relative bone content of carcasses may be comparatively easily estimated using metapodial median lengths as long as animals are hunted during the same season in a defined area. As opposed to these two components, carcass fat percentage is visibly less in males than in females. Quantification, however, requires this hypothesis to be tested using more numerous dissection data. Sexual dimorphism, on the other hand, should be taken into consideration when carcass weight itself is being predicted due to the sex-related differential growth of most tissues under the influence of sexual hormones. This effect was equally detected in the long bone growth of cattle and elk (Bartosiewicz, 1984; 1985; 1987*a*) as well as in the cranial proportions of elk from Scandinavia (Saether, 1983; Bartosiewicz, 1987*c*). This influence increases with the advancement of sexual maturation. While the endocrinological aspect of this problem was outlined in detail previously (Bökönyi and Bartosiewicz, 1983) this mechanism may be interpreted very much the same way as is the case with malnutrition (Pálsson, 1955) or pathological stress (Stamp, 1982). Tissues displaying intensive growth represent a *locus minoris resistentiae* in the body and thus are more prone to distorsion by both external and internal effects.

This critical period in skeletal growth could well be observed in the body formation of female cattle between 120 and 225 kg of live weight (Bartosiewicz, 1987*d*). When factor scores of the second factor analysis are plotted against each other, a clear divergence may be seen between the body conformation of female and male elk along the axis representing age (Fig. 3). In spite of the relatively clear dichotomy between sexes, however, the pattern of age is not very clear. Sex-related effects in the ontogenetic model presented here may be removed by using carcass weight as independent variable instead of Factor 1, age. This latter also contains "noise" from the overlap with Factor 2 i.e. sex. The resulting curves are shown in Fig. 4. Tendencies obtained for both sexes are reminiscent of the cosine function. Their

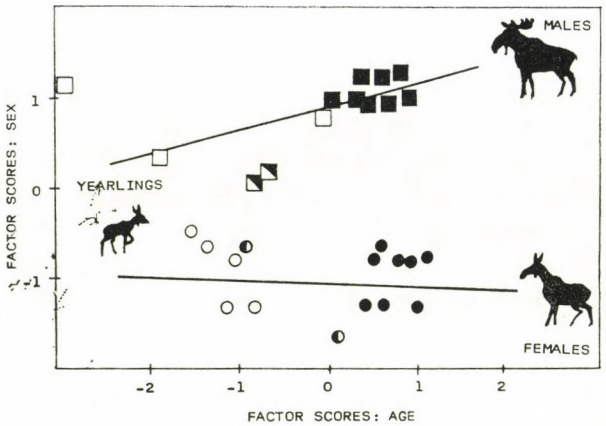

Fig. 3. Divergent trends outlined by 20 males (squares) and 16 females (circles) simulated in the plane of the two factors describing carcass composition traits (Table VII). The number of data points shown is reduced by overlaps between cases. White units show individuals with unfused distal metapodial epiphyses. Shading indicates complete fusion. Transitional forms are marked by both colors

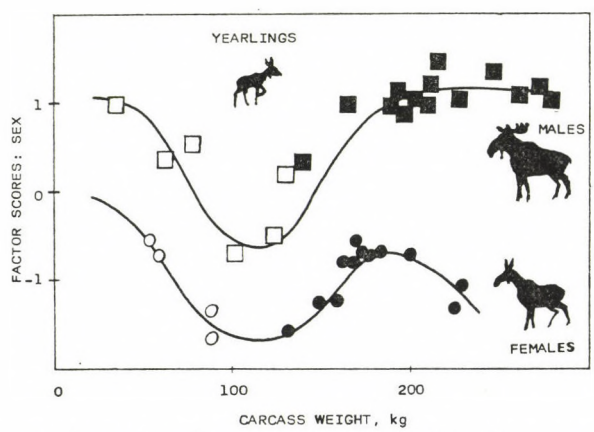

Fig. 4. Factor scores of sex plotted against carcass weight to show changes in the sex-dependent components of the variables listed in Table VII. The minimum point in both curves and the divergence of tendencies following sexual maturation are of particular interest. For legend see Fig. 3

exact mathematical definition, however, would edge on the overexploitation of nothing but simulated data. It is much more important that both curves have their deepest point in exactly the same carcass weight interval which was pinpointed in previous examples (100 to 120 kg) by various kinds of physiological evidence independent of the results of this study (epiphyseal fusion, intersection between female and male growth curves, sexual maturation etc.). Increasing sexual dimorphism beyond this period shown by the progressive divergence of sex-related traits encompassed by Factor 2 (sex) may even be more clearly seen here than in Fig. 3.

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TUBULAR REABSORPTION OF PROTEIN BY PORCINE KIDNEYS DURING NEONATAL ALIMENTARY PROTEINURIA

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The ability of renal proximal tubular cells to reabsorb protein early in postnatal life was investigated using goat haemoglobin as tracer. The haemoglobin was intracardially administered to newborn piglets. The kidneys were fixed for light and electron microscopy 4 h later. Piglets killed immediately after birth and those allowed to suck colostrum for 4 h were used as controls.

The proximal tubular cells of newborn, unsuckled piglets already contained absorptive vacuoles. Haemoglobin was absorbed in some absorptive vacuoles of the proximal tubules. The tracer was also demonstrable in the urine, mainly in the form of methaemoglobin.

Although proximal tubular cells of newborn piglets are able to absorb protein, this absorption is of limited extent and excess protein is voided with the urine.

Keywords: Pig, newborn, proteinuria, kidneys, proximal tubules.

Ungulates are born almost completely agammaglobulinaemic; passive immunity is acquired by the intestinal absorption of colostral antibodies (Brambell, 1970) which mainly belong to the IgG class. In contrast to rats and mice, this absorption is non-selective in ungulates, i.e. apparently every protein species of the colostral whey and their proteolytic fragments are absorbed into the neonatal circulation during the first one or two days of life (reviewed by Baintner, 1986). The osmotic load of large quantities of absorbed protein (McCance and Widdowson, 1959; Ramirez et al., 1963) is alleviated by urinary excretion of the smaller colostral proteins and protein fragments passing through the glomerular filter of the kidneys, while intact antibodies remain in the circulation. The physiological, alimentary proteinuria (Howe, 1924; Smith and Little, 1924; McDougall, 1965; Loh et al., 1972; Martinsson, 1973) ceases soon after closure of intestinal absorption of macromolecules, usually during the second day of life. Beta-lactoglobulin (Pierce, 1961*a*, *b*). colostral trypsin inhibitor (Baintner, 1970) and IgG fragments (Kickhőfen et al., 1972; Martinsson, 1972) were identified in the urine of ungulates after the first suckling. Even haemoglobin was excreted by the kidneys, if fed mixed with colostrum (Baintner, unpublished observation).

It is known that in adult mammals small amounts of serum protein escape continuously from the circulation into the primary urine and are taken

up by the tubular epithelium, mainly in the proximal tubules. After intracellular digestion the amino acids are reutilized.

The question arises whether the protein, which appears in the piglet's urine, is in excess of tubular absorptive capacity or whether reabsorption of protein does not function early in postnatal life. The present study supports the first alternative.

Materials and methods

Animal and experimental design

Twelve newborn KAHYB hybrid piglets of both sexes, weighing 1.20–1.30 kg, out of four litters, were used.

Immediately after parturition three piglets were marked and selected for the experiment. One piglet was killed at about one hour after birth (unsuckled control); another piglet was allowed to suck colostrum from the dam (suckled control), together with littermates not included in the experiment; a third piglet was not allowed to suck, but was injected with goat haemoglobin as tracer protein (haemoglobin-treated piglet). The suckled control and the haemoglobin-treated piglets were killed by bleeding at about 4 h of age, 3 to 4 h after the first suckle or the injection of haemoglobin, to allow time for the proteins to reach the renal tubules and to be absorbed. The experiments were repeated with piglets of three other litters. Blocks (approximately one mm^3) were excised from the subcapsular region of the kidneys for electron microscopy and 0.5 cm thick slices for light microscopy.

Preparation and administration of haemoglobin

Heparinized goat blood was centrifuged at about 1000 *g* for 10 min and the supernate was discarded. The red blood cells were washed three times with saline then haemolysed with distilled water. Tonicity was restored to physiological by adding a 9 : 1 mixture of concentrated NaCl and KCl solutions. The haemoglobin solution was administered *intracardially* under ether anaesthesia because of difficulties of intravenous administration. The heart was located by palpation on the left side of the thorax. After inserting the needle in the right angle between the ribs, 1 to 2 ml blood was removed, then 0.5 g haemoglobin was slowly injected in 6 ml solution. The treated piglets were kept under an infrared lamp.

Preparation of tissue samples

Specimens fixed in 8% formaldehyde (adjusted to pH 7.4 with phosphate buffer) were embedded in paraffin and stained with haematoxylin and eosin (HE). Haemoglobin, as a tracer, was made visible by the diaminobenzidine method of van Duijn (1955).

One mm^3 blocks of renal tissue were immersed in 4 °C % glutaraldehyde in 0.15 M Na-cacodylate, pH 7.4 for 4 h, and washed in 4 °C 0.1 M phosphate buffer, pH 7.4, for 2 h, then postfixed in 2% OsO_4 (in 0.1 M, pH 7.4 phosphate buffer) for 1 h. Following dehydration in ethanol, the specimens were embedded in Durcupan ACM (Fluka). The peroxidase reaction was carried out according to van Duijn (1955) on 1 μm sections, then stained with toluidine blue. Ultrathin sections were stained with uranyl acetate and lead citrate or treated with diaminobenzidine to detect peroxidase activity according to Robbins et al. (1971).

Urinary proteins

Urine was taken from the bladder of the killed piglets. The presence of urinary proteins was checked by precipitation with trichloroacetic acid. Urinary spectra were determined with a Beckman UV 5260 spectrophotometer in visible and ultraviolet light at the natural pH (6.0 to 6.5) of the urine. If necessary, the samples were cleared by centrifugation. The spectrum of goat haemoglobin solution was determined for comparison.

Results

Light microscopy and transmission electron microscopy showed glomeruli of fetal type (without podocytes) and mature tubules with open lumina. In the electron microscope, the proximal tubules were identified by the presence of cells with long microvilli on the luminal side. Small vesicles and tubules ($\sim$ 0.1 μm in diameter) were seen in the apical part of the proximal tubular cells (Fig. 1) and relatively large vacuoles (1.0 to 2.5 μm) in other regions of the cell (Fig. 1). Most of the vacuoles contained fine granular material with higher density than that of the basement membrane. Vacuolar contents showed heterogeneity with clumps of higher electron density at the inner side of the limiting membrane. Similar vacuoles were seen in all groups of piglets. Clear differences in electron density of the vacuolar contents between haemoglobin-treated and untreated piglets were not observed.

Red blood cells and granulocytes gave a positive peroxidase reaction both in paraffin-embedded and ultrathin sections of all kidneys. In sections

of paraffin-embedded specimens from haemoglobin-treated piglets a brown product of the peroxidase reaction was seen on the cells of the proximal convoluted tubules. The distal tubules gave a weaker reaction and in the pars recta of the proximal tubules no reaction product was found. In the 1-μm thick and ultrathin sections the electron-dense peroxidase product was localized on some of the absorptive vacuoles of the tubular cells in the haemoglobin-treated piglets (Fig. 2) and, less markedly, in the intracellular space (Fig. 3). Tubules of untreated newborn piglets gave no peroxidase reaction. The lack of peroxidase-active material in the tubular lumina may be explained by the extraction of the primary urine during fixation and preparation of the specimens.

Piglets had various quantities of urine in the bladder when killed. The haemoglobin-treated and the suckled control piglets contained trichloroacetic acid precipitable material in the urine whereas the unsuckled control piglets did not. Urine samples of the haemoglobin-treated piglets were brown to a different extent and the darkest samples were made turbid by a brown precipitate. Spectra in visible light demonstrated the presence of methaemoglobin and of a minor fraction of oxyhaemoglobin (Fig. 4).

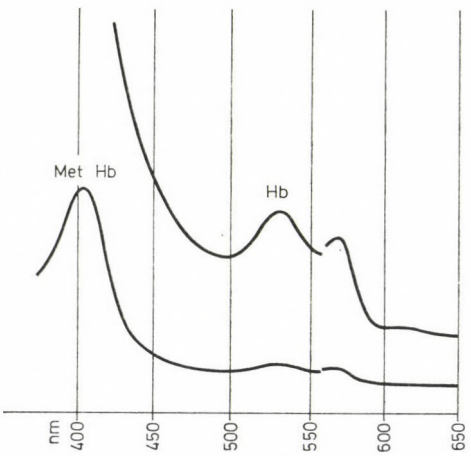

Fig. 4. Light absorption spectrum of the urine of a newborn piglet injected with goat haemoglobin 4 h before it was killed. The spectrum was determined between 350 and 650 nm with two different concentrations of urine and it shows peaks characteristic of methaemoglobin and oxyhaemoglobin

Discussion

It is known that the tetrameric haemoglobin molecule dissociates to dimers and monomers if it is released from its concentrated solution in the erythrocytes, and so it becomes capable of passing through the glomerular filter.

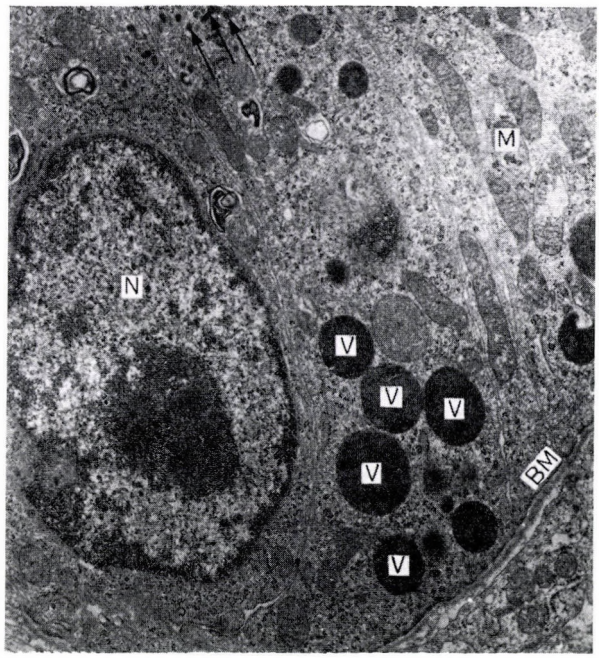

Fig. 1. An epithelial cell of a proximal convoluted tubule in a haemoglobin-treated piglet. In the basal part of the cell a group of absorption vacuoles (V) with heterogeneous content are seen. In the apical part of the cell small vesicles contain electron-dense material (arrows). The vacuoles and vesicles are similar to those of the control animals. N = nucleus; M = = mitochondria; BM = basement membrane. ×12,000

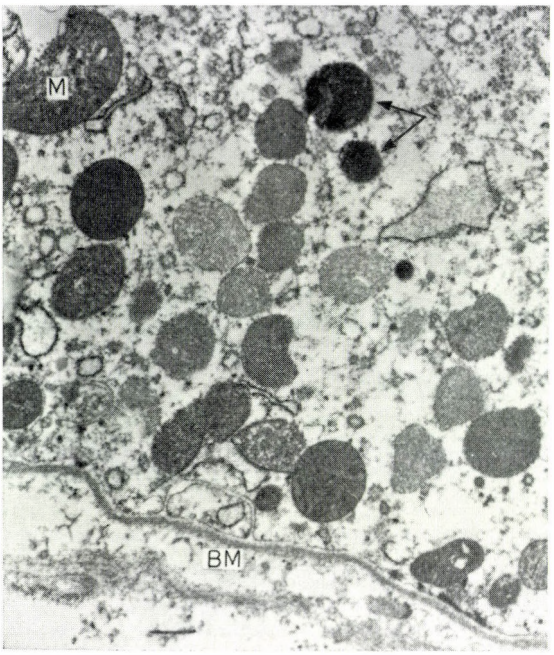

Fig. 2. Absorptive vacuoles (V) in the basal part of an epithelial cell of a proximal convoluted tubule in a haemoglobin-treated piglet. Electron-dense peroxidase reaction product indicates the presence of absorbed haemoglobin tracer in some vacuoles (arrows). M = mitochondria; BM = basement membrane. Diaminobenzidine reaction, ×12,000

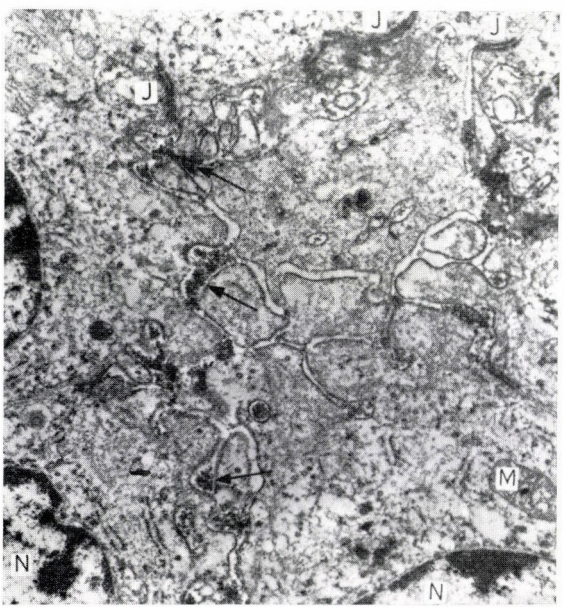

Fig. 3. Epithelial cells of a proximal convoluted tubule in a haemoglobin-treated piglet. Electron-dense reaction product (arrows) is located in the intercellular spaces below the junctional complexes (J). N = nucleus; M = mitochondria. Diaminobenzidine reaction, ×12,000

Proteinuria due to colostral protein in the suckled control piglets and methaemoglobinuria in the haemoglobin-treated piglets indicated that proteins reached the kidneys from the gastrointestinal tract or the site of injection, respectively, before the piglets were killed. Most of the haemoglobin tracer was denatured to methaemoglobin during excretion.

Although the renal function of piglets is still developing in the first postnatal weeks (Friis, 1979, 1980), the ultrastructure of the convoluted tubules suggested an adult-like function in the newborn piglet: they contained absorptive vacuoles in the tubular cells. Unexpectedly, these vacuoles were already present before the first suckle and showed similar contents subsequently. The function of absorptive vacuoles is not quite clear at this early age; it possibly indicates uptake and digestion of serum proteins that have escaped from the circulation through immature glomeruli.

The presence of absorptive vacuoles in the unsuckled control piglets made conventional transmission electron microscopy useless for tracing the reabsorption of colostral protein or haemoglobin in the experimental piglets. Uptake of haemoglobin by the proximal tubular cells was indicated by the positive peroxidase reaction in the haemoglobin-treated piglets, as shown by paraffin-embedded, 1 μm thick and ultrathin sections.

It is concluded that newborn piglets are able to reabsorb the haemoglobin tracer and therefore alimentary proteinuria is primarily due to saturation of tubular absorptive capacity, rather than to immaturity of renal function. The newborn piglet may be able to utilize a certain fraction of low molecular weight colostral protein by reabsorption and digestion in the renal tubular epithelium. Adult and neonatal reabsorptive capacity of the renal tubules was not compared in the present study.

Acknowledgements

We would like to thank Mrs Ilona Deák for the preparation of specimens for electron microscopy and the pig farm of Nagyberki, personally to Mr Z. Zeller, for providing piglets and assistance for the experiment.

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ACUTE TOXICOLOGICAL EXPERIMENT OF T-2 TOXIN IN RABBITS

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Rabbits were treated with a single oral dose (1, 2, 4, 6, 8, 10 or 15 mg/kg body mass) of T-2 fusariotoxin. Doses of 4 mg or higher killed the animals in 24 to 48 h. As opposed to the controls, in the treated rabbits gross pathological and histopathological examinations revealed acute catarrhal gastroenteritis, necrosis of lymphoid cells of the gastrointestinal mucosa, centrolobular dystrophy of the liver, necrosis of cells of the mononuclear phagocyte system (MPS) in the liver, tubulonephrosis, focal dystrophy of the adrenal cortex, lymphocyte depletion involving both T- and B-cell-dependent zones of the lymphoid organs (spleen, lymph, ampulla ilei), and depletion and necrosis of the myelopoietic cell colonies of the bone marrow. Similar but milder changes were observed in surviving rabbits exsanguinated 48 h after treatment.

In addition to the direct damage done to the digestive tract mucosa and liver, the toxin severely damaged the cells participating in humoral and cell-mediated immunity and in the local defence of the intestinal mucosa, and markedly impaired phagocytosis and granulocytopoiesis.

In another experiment rabbits were given oral doses of 2 mg/kg body mass T-2 toxin daily for several days. One rabbit was killed by bleeding every day. In rabbits killed beyond day 7 there was subacute catarrhal gastritis, emaciation, and hypertrophy of the adrenal cortex.

Keywords: T-2 mycotoxin, fusariotoxicosis, rabbit, pathology.

In two cases of T-2 mycotoxicosis among broiler chicks necrosis of the oral and oesophageal mucosa and atrophy of the bursa of Fabricius, thymus and spleen were detected (Bitay et al., 1979, 1981). The lesions produced by T-2 toxin in mammals include congestion of the meningeal vasculature, severe congestion of the jejunal and ileal mucosae, lesions in the jejunum, ileum, caecum, spleen and mesenteric lymph nodes (karyopyknosis, karyorrhexis), Peyer's patches of the ileum, lymphoid elements of the caecum, lymphoid follicles of the spleen, and germinal centres of the mesenteric lymph nodes (Sato et al., 1975; Weaver et al., 1978; Hayes et al., 1980; Corrier and Ziprin, 1986; Pang et al., 1986; Glávits and Ványi, 1988).

The purpose of this experiment was to study the pathological changes produced by T-2 fusariotoxin in rabbits.

Materials and methods

Thirty-nine clinically normal rabbits, weighing 2.4–2.6 kg, were used. In an acute toxicological experiment, 14 rabbits were given 1–15 mg/kg body weight T-2 fusariotoxin in dimethyl sulfoxide (DMSO) solution on one occasion, per os, through a gastric tube. Four animals received only DMSO, without T-2 toxin, and served as controls. They were examined clinically and the survivors and controls were exsanguinated 48 h after treatment. In a subacute toxicological experiment 15 rabbits received in the feed 2 mg/kg body weight T-2 fusariotoxin daily. The rabbits were killed by bleeding two, or in some cases one, at a time 6, 12, 20 and 48 h, and 3, 4, 5, 6, 7, 8, 9, and 10 days after treatment. The 6 controls were exsanguinated 20 and 48 h, and 4, 6, 8, and 10 days after treatment, respectively.

Histological examination. Samples were taken from the liver, spleen, kidney, adrenal gland, stomach, small intestine, ampulla ilei, mesenteric lymph node and bone marrow of each animal. After fixation in 5% buffered formalin and embedding in paraffin, the sections were stained with haematoxylin and eosin. In frozen sections prepared from the liver, Fettrot staining was used for the detection of lipids.

Electron microscopic examination. Spleen and bone marrow samples were taken from two treated (8 mg/kg body weight) and two control animals of the acute toxicological experiment. After double fixation in 2.5% glutaraldehyde and in 1% osmium tetroxide, both buffered with 0.1 M sodium cacodylate, and dehydration with ethanol, the samples were embedded in Durcupan ACM in the usual way. Ultrathin sections were counterstained with uranyl acetate and lead citrate, and examined in a Philips 201 CS electron microscope.

Results

The results are shown in Tables I and II and in Figures 1–7.

Conclusions

Accumulation of serous exudate in the thoracic and abdominal cavity is indicative of disturbed circulation. The acute or subacute catarrhal gastritis and enteritis may be a consequence of the direct cytopathic effect exerted by the toxin. The damage done to lymphoid cells of the intestinal mucosa is suggestive of a suppressed local immune response, whereas lesions seen in the B- and T-cell-dependent zones of the lymphoid organs are indicative of suppressed humoral and cell-mediated immune responses. Necrosis of cells be-

Table I

Pathomorphological changes in rabbits caused by T–2 toxin (acute toxicological experiment, 1–15 mg/kg body weight/one occasion, p. o.)

Pathologic changes		Toxin dose (mg/kg body mass)													
		1	1	2	2	4	4	6	6	8	8	10	10	15	15
Death							+	+	+	+	+	+	+	+	+
Changes	serum accumulation in the thoracic and abdominal cavity						+			+				+	+
(48 h after treatment)	hyperaemia of lungs, emphysema and/or oedema	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	acute gastritis						+	+	+	+	+	+	+	+	+
	acute enteritis						+	+	+	+	+	+	+	+	+
	lymphoid cell necrosis of the intestine*				1	2	2	3	2	3	3	3	3	3	3
	pathological fatty liver degeneration				+		+	+	+	+	+	+	+	+	+
	necrosis of liver MPS cells*					1	2	2	2	2	3	3	3	3	3
	tubulonephrosis			+		+	+	+	+	+	+	+	+	+	+
	haemorrhage and reduction of lipid content in the adrenal cortex		+		+		+		+	+	+	+	+	+	+
	lymphocyte depletion and necrosis in the "B" and "T" dependent zones of the spleen and lymph nodes and lymphoid follicles of the ampulla ilei*			1	1	2	2	2	2	3	3	3	3	3	3
	blast cell depletion and necrosis in bone marrow myeloid colonies*			1	2	1	2	2	2	3	3	3	3	3	3

*1 — up to 5–30% of the cell population
2 — up to 30–80% of the cell population
3 — up to 80–100% of the cell population

Table II

Pathologic changes in rabbits caused by T–2 toxin (subacute toxicological experiment, 2 mg/body weight daily, p. o.)

Pathologic changes	Post-treatment hours and days until killed													
	hours						days							
	6	12	20	20	48	48	3	4	5	6	7	8	9	10
loss of weight												+	+	+
gastritis								+	+	+	+	+	+	+
lymphocyte necrosis of small intestine mucosae*	1	1	1	1	1	1	1	1	1	1	1	1	1	1
lymphocyte depletion and necrosis in the lymphoid follicles of ampulla ilei*	1	1	1	1	1	1	1	1	1	1	1	1	1	1
centrolobular pathological fatty liver degeneration			+		+									
adrenal cortex hypertrophy											+	+	+	+
lymphocyte depletion and necrosis in the "B" and "T" dependent zones of the spleen and lymph nodes	1	1	1	1	1	1	1	1	1	1	1	1	1	1
blast cell depletion and necrosis in myeloid colonies of the bone marrow*	1	1	1	1	1	1	1	1	1	1	1	1	1	1

* 3–10% of the cell population is altered

longing to the MPS (mononuclear phagocyte system) indicates disturbed phagocyte function, and impaired granulocytopoiesis in the bone marrow reflects an impaired natural resistance (i.e. inflammatory responsiveness). The degenerative changes found in the parenchymal cells of the liver and kidneys may be connected with the systemic effect of the toxin, whereas the lesions observed in the adrenals may be attributed, at least in part, to effects exerted by stressors.

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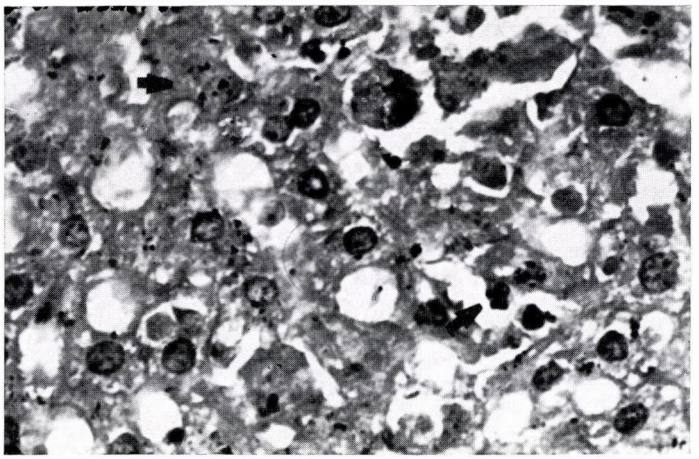

Fig. 1. Detail of liver from a rabbit that died 24 h after oral treatment with 10 mg/kg body mass T-2 toxin. Note vacuolar degeneration of hepatocytes some of which have become necrotic, rounded off and left the cell row. Cells of the mononuclear phagocyte system (MPS) are consistently necrotic and show karyorrhexis and karyopyknosis with only necrotic remnants of the nucleus left behind (arrow). Haematoxylin and eosin (H-E.), ×400

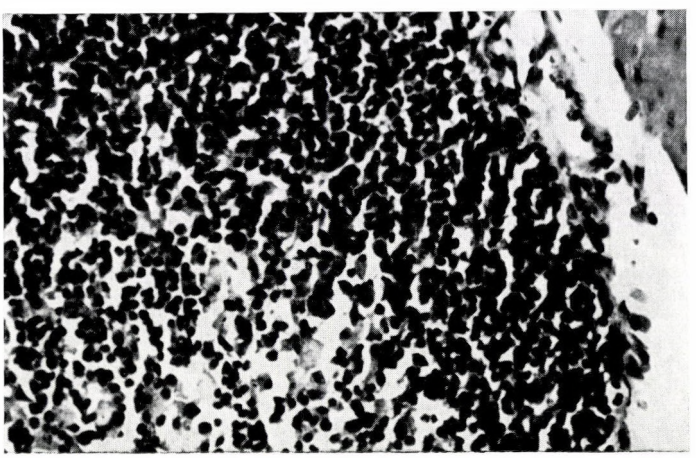

Fig. 2. Detail of ampulla ilei from an untreated control rabbit. Note large numbers of dark-staining intact lymphocytes in the cortex, and less such cells in the medulla, of the lymphoid follicle. H-E., ×160

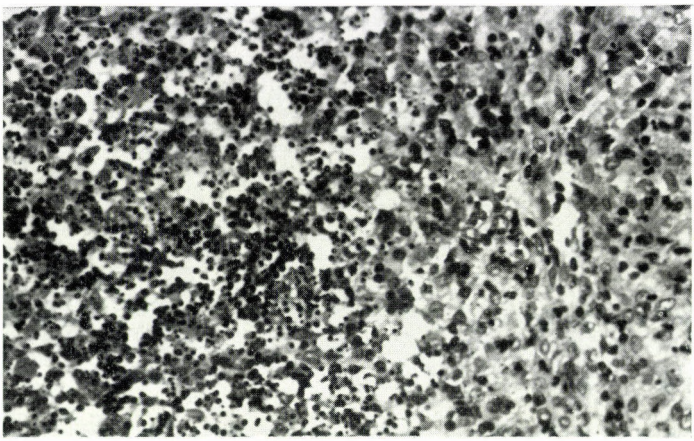

Fig. 3. Detail of ampulla ilei from a rabbit that died 24 h after oral treatment with 8 mg/kg body mass T-2 toxin. Note marked lymphocyte depletion in the cortex and necrotic lymphocytes with shrunken or disrupted nuclei as well as structural disintegration in the medulla of the lymphoid follicle. H-E., ×160

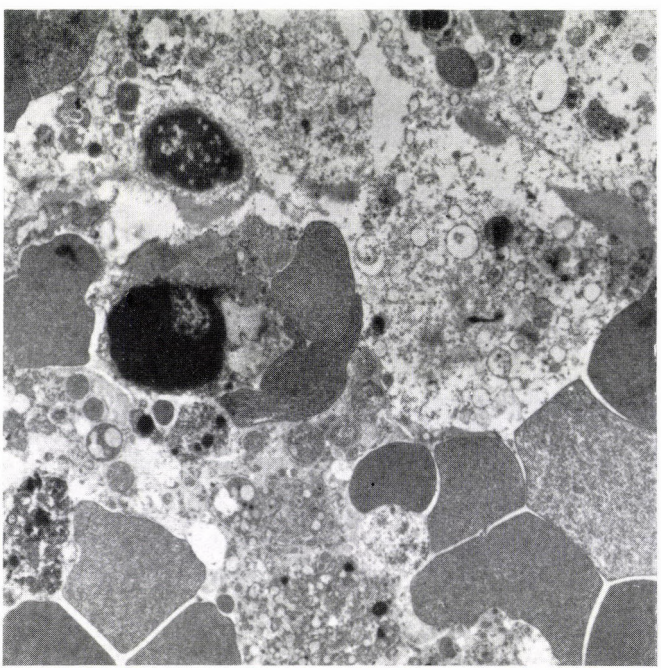

Fig. 4. Spleen from a rabbit that died 24 h after oral treatment with 8 mg/kg body mass T-2 toxin. In the area of the Malpighian corpuscles note remnants of necrotic, disrupted lymphocytes along with red blood cells. Electron micrograph, ×6400

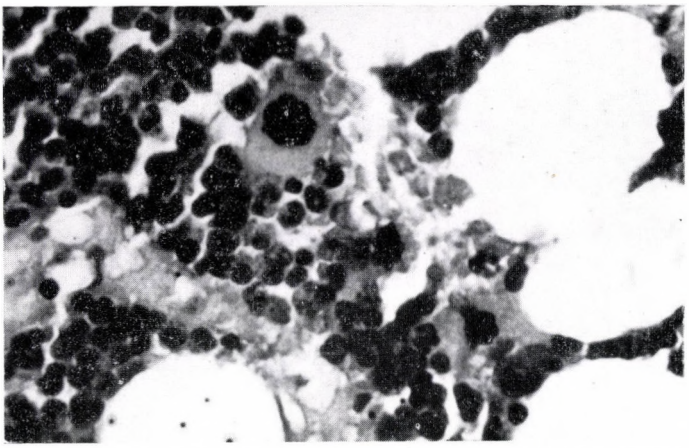

Fig. 5. Detail of bone marrow from an untreated control rabbit. Note immature stages of myeloid and erythroid haemocytopoiesis (blast cells) and a giant cell (megakaryocyte). H-E., ×400

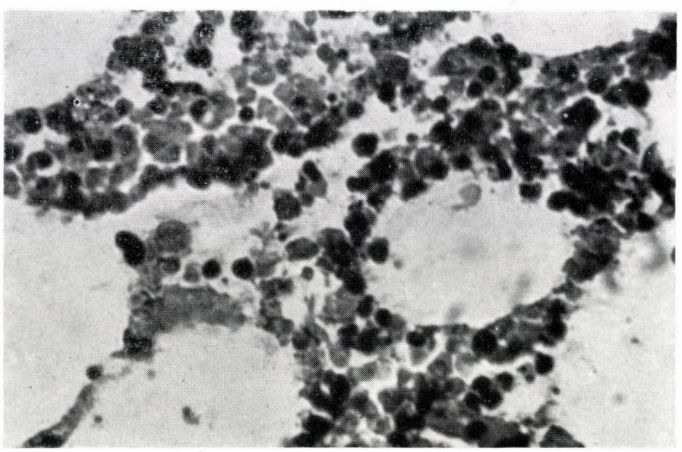

Fig. 6. Detail of bone marrow from a rabbit that died 24 h after oral treatment with 8 mg/kg body mass T-2 toxin. Myeloblasts and erythroblasts have decreased in number and those that remained show signs of degeneration or necrosis (karyopyknosis or karyorrhexis). H-E., ×400

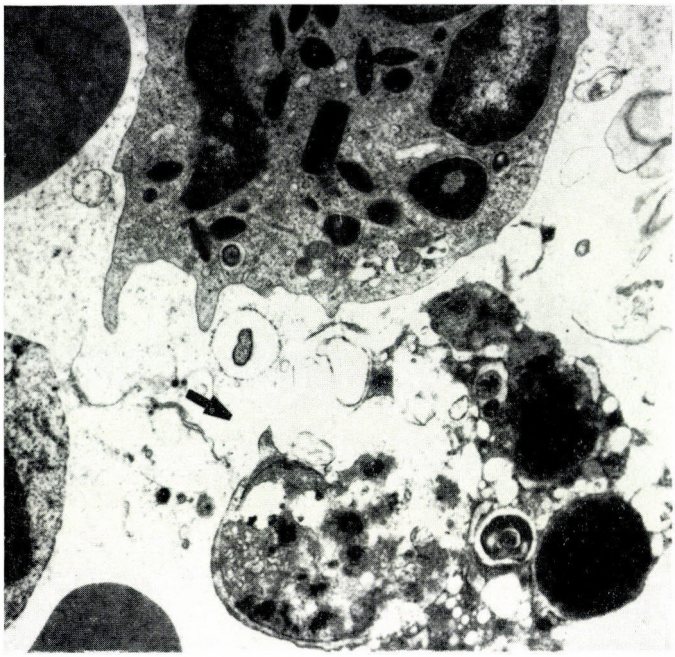

Fig. 7. Bone marrow from a rabbit that died 48 h after oral treatment with 4 mg/kg body mass T-2 toxin. Note necrotic, disintegrated myeloblast (arrow) and myelocyte. Electron micrograph, ×14,300

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ULTRASTRUCTURAL OBSERVATIONS ON DIFFERENT DEVELOPMENTAL STAGES OF *GOUSSIA SINENSIS* (CHEN, 1955), A PARASITE OF THE SILVER CARP (*HYPOPHTHALMICHTHYS MOLITRIX* VALENCIENNES, 1844)

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The development of *Goussia sinensis*, a coccidium parasitizing the intestine of the silver carp *(Hypophthalmichthys molitrix)* was studied by electron microscopy. All stages developed in the epithelial cells, less frequently in the goblet cells, and were located within a parasitophorous vacuole. In some cases one cell was invaded by several merozoites. Eight to sixteen merozoites were formed within the meront by ectomerogony. The ultrastructural processes characteristic of gamogony were the same as those found for *Goussia* spp. parasitizing other species of fish. A hitherto unknown mechanism of oocyst wall formation was observed. The oocyst membrane developing within the zygote surrounded only part of the zygote material. Thus, a small part of the zygote material left the oocyst proper. It is suggested that this zygote residue and the necrotic host cell constitute the so-called "yellow bodies" which include the excreted oocyst. The oocyst wall was 40 to 60 nm thick. Oocyst sporulation took place within the fish. The sporocysts consisted of two hemispheres connected by sutures and had a 100 to 120 nm thick double wall. They were surrounded by sporocyst veils fixed to the oocyst wall by membranes.

Keywords: *Goussia sinensis*, ultrastructure, developmental stages, parasite, silver carp, *Hypophthalmichthys molitrix.*

Little is known about the ultrastructure of fish parasitic coccidia. Researchers studying the structure of oocysts (Lom, 1971; Odense and Logan, 1976; Davies, 1978; Morrison and Hawkins, 1984) consistently concluded that the oocyst and sporocyst wall of fish coccidia differed in structure from those of coccidia parasitizing mammals and birds. At the same time, merogony and gamogony are similar in fish and in other animal species (Duszynski et al., 1979; Hawkins et al., 1983*a;* Hawkins et al., 1983*b*, 1983*c;* Paterson and Desser, 1981*a*, 1981*b*, 1981*c;* Desser and Li, 1984). A different development has only been observed by Molnár and Baska (1986) and Paperna and Landsberg (1987) in *Epieimeria anguillae* and *Eimeria vanasi* respectively, which have extracytoplasmic merogony and gamogony resembling those of Cryptosporidia.

The species *Goussia sinensis* was originally described in China by Chen (1955) as *Eimeria sinensis*. Later, however, on the basis of its characteristic

bivalve sporocyst shell Dykova and Lom (1983) assigned this parasite into genus *Goussia* Labbé, 1896. *G. sinensis* was first reported to occur in Europe after the silver carp *(Hypophthalmichthys molitrix)* and bighead *(H. nobilis)*, known as its host species, had been introduced (Musselius, 1967). The host specificity of *G. sinensis* was reviewed by Musselius and Laptev (1967) and its development was discussed by Molnár (1976).

In the present work *G. sinensis* infection of silver carp cultured in a farm-pond was monitored by electron microscopy. The ultrastructural characteristics of the formation of merogonic stages, microgamonts, macrogamonts and oocysts, different from those reported earlier, are described.

Materials and methods

The test material consisted of 2 to 3 months old silver carp obtained from a farm-pond and naturally infected with *G. sinensis*. The fish were examined in August 1985. According to Molnár (1976), the cultured fry regularly develop an infection of high intensity in August. The fish were killed 6 h after having been caught, and samples were taken from different segments of the gut for electron microscopic examination. One-mm^3 pieces were excised from the gut mucosa, fixed in 5% glutaraldehyde dissolved in 0.13 M sodium cacodylate buffer at 4 °C for 2 h, washed with 0.13 M sodium cacodylate and post-fixed in 1% osmium tetroxide. After dehydration and embedding in Durcupan ACM resin, sections were counterstained with uranyl acetate and lead citrate.

Parallel to the electron microscopic examinations, the gut segments were processed for histological study by light microscopy, in order to select the most severely infected segments for electron microscopy.

Results

All *G. sinensis* developmental stages found in the present study (from the youngest trophozoites to sporulating oocysts) were located in the gut epithelial cells apical to the nucleus. Trophozoites surrounded by a separate parasitophorus vacuole in the cytoplasm of the gut epithelial cells were considered the youngest developmental stages (Figs 1 and 7). In some cases several (10–12) of these elongated trophozoites occurred within a single epithelial cell (Fig. 1). These trophozoites were probably derived from merozoites rather than from sporozoites. Parasitized cells were elongated in shape, had an intact brush border, a distinct nucleus and granulated cytoplasm containing numerous mitochondria.

The merozoites developed by exogenesis (Fig. 2) during which most of the cytoplasm was used up, leaving behind little or no residual material. There were usually 8 or 16 merozoites within a single vacuole (Figs 3 and 4). The merozoites were bordered by a 3-layered pellicle under which 22 subpellicular tubules were seen in cross section (insert of Fig. 4). Occasionally well-formed micropores were seen in the pellicle. The apical cross sections of the merozoites showed numerous micronemes and rhoptries. In longitudinal sections of the merozoites the characteristic conoid structure, the basally located large nucleus, tubular mitochondria, abundant endoplasmic reticulum, and extremely large numbers of ribosomes were seen. The epithelial cell containing the merozoites underwent considerable enlargement and the parasitophorous vacuole was surrounded by a ring-shaped cytoplasm. The cytoplasm was separated from the vacuole by a distinct single-layered unit membrane. In some cases merogony took place also in the goblet cells (Fig. 5), giving rise to merozoites the number of which was the multiple of four.

The microgamont (Fig. 6) was also located in a parasitophorous vacuole and contained numerous nuclei with heterochromatin arranged along the nuclear membrane. The nuclei of the microgamonts gradually assembled at the periphery and were situated immediately under the unit membrane of the gamont. They developed into microgametes and the cross-sections of their perforatoria and flagella were seen freely in the lumen of the parasitophorous vacuole (Fig. 7).

The young macrogamont (Fig. 8) also developed within a parasitophorous vacuole and its cytoplasm contained, besides the nucleus, numerous lipid and amylopectin granules. The parasitophorous vacuole was demarcated from both the host cell and the macrogamont by a single-layered basal membrane. In some preparations it was well visible that intact epithelial cells had closed around the inactive cell containing the macrogamont (Fig. 9). Thus, the developing oocyst was pushed towards the deeper layers.

A hitherto unknown mechanism of oocyst wall formation has been discovered. At the beginning of zygote division the lumen of the parasitophorous vacuole was almost completely filled by the parasite and, thus, it virtually disappeared. Along the inner membrane of the parasitophorous vacuole a portion of the zygote cytoplasm, having electron-dense matrix and containing amylopectin and lipid granules, separated, while centrally an oocyst surrounded by a distinct unit membrane developed (Figs 10 and 11). The cytoplasm portion that had been eliminated from the oocyst attached itself to the oocyst surface as an external residual body at one or two segments, while less frequently it entirely surrounded the oocyst. In some cases even this residual body had become very thin. The oocyst wall was a single 40 to 60 nm thick membrane.

The sporulation of oocysts took place within the fish. Division of the sporont gave rise to four sporoblasts, each containing two sporozoites and a

residual body (Fig. 10). The sporocyst wall consisted of two hemispheres connected by sutures (Fig. 12). The sporocyst wall was 100 to 120 nm thick and consisted of two layers. The outer layer was 12 to 16 nm thick and had a membrane-like structure, whereas the inner homogeneous layer was 80 to 100 nm thick (Fig. 12). The sporocysts were loosely surrounded by a sporocyst veil (Fig. 13) which was in some places connected with the oocyst wall by membranes. At the same time, the sporopodia starting from the sporocyst surface and supporting the soprocyst veil in some fish parasitic coccidia were absent from the sporocyst of *G. sinensis*. Due to the fixation technique applied by us, in older oocysts the sporocysts were shrunken and the oocyst wall detached itself from its surroundings (Fig. 13).

Discussion

Knowledge of the development of coccidia within the fish is mostly based on observations, since only few researchers (Paterson and Desser, 1981*a*, *b*, *c;* Hawkins et al., 1983*b*, *c)* have studied the various developmental stages in experimentally infected fish. The present work is also based on natural infection where youngest merogonic stages are especially difficult to study since there are rather few of them.

The present studies do not allow us to decide whether the youngest trophozoites newly entering the cells have developed from sporozoites or from merozoites. Neither can it be determined whether these trophozoites will develop into second- or third-generation meronts or into gamonts. In this respect location within the cells is not informative, as both the merogonic and the gamogonic stages are situated in the cytoplasm near, and apical to, the nucleus of the epithelial cell. The youngest motile stages invading the host cell are immediately surrounded by a parasitophorous vacuole.

In most cases a single trophozoite developed within a cell. Occasionally, however, a single cell contained 10 to 12 trophozoites, each having a separate parasitophorous vacuole. We were unable to collect data on the further development of these trophozoites: probably the host cells invaded by such a large number of trophozoites became necrotic and were expelled.

It was reported by Molnár (1976) for *G. sinensis* that in most cases a meront gave rise to 16 merozoites; however, units comprising 8 merozoites within the infected host cell also occurred. Our electron microscopic studies have confirmed this observation: in the majority of cases meronts consisting of 8 or 16 merozoites were seen. From the present studies it cannot be determined how many merogonies take place during the developmental cycle. Neither can we tell whether the emergence of meronts consisting of 8 or 16 merozoites corresponds to a stage of merogony or, rather, the fact is that during the mero-

gony of *G. sinensis* the number of merozoites is not always the same. Starting from the fact that meronts consisting of 16 merozoites are much more frequent, it can be assumed that units consisting of 8 merozoites represent an earlier stage of merogony. Regular development of 8 or 16 merozoites could suggest development by endodyogeny; however, from Fig. 2 it is clear that *G. sinensis* develops by ectomerogony, similarly to that reported for *G. iroquoina* and *G. funduli* (Paterson and Desser, 1981*c*; Hawkins et al., 1984).

The formation of microgamonts takes place as described by Desser and Li (1984) and Molnár and Baska (1986): i.e. microgametes are formed ectoplasmally from nuclei pushed to the surface of the microgamont.

The fate of the parasitized host cell depends on the developmental stage invading it. Host cells containing meronts and microgamonts stay on the surface until the merozoites and microgametes leave them. On the other hand, as it was suggested by Molnár (1984), some of the cells containing macrogamonts are unable to perform their covering function, and the surrounding intact epithelial cells overgrow them and finally push them to deeper layers. Although the macrogamont is also located apical to the nucleus, oocyst formation takes place in the deep layers and not on the surface of the gut epithelium.

Oocyst wall formation deserves particular interest. In coccidia of mammals and birds the oocyst wall is formed by the so-called wall-forming bodies appearing in the sporont (Chobotar and Scholtyseck, 1982). In the case of *G. sinensis* the oocyst wall is also formed by the sporont, with the exception that no wall-forming bodies are demonstrable in the sporont which could participate in forming the extremely thin oocyst wall. This observation is inconsistent with the findings of Hawkins et al. (1983*a*) who reported that the oocyst wall of *Calyptospora funduli* originated from the host cell unit membrane surrounding the parasitophorous vacuole. Sporocysts of *G. sinensis* are also loosely surrounded by a sporocyst veil which is connected with the oocyst wall by supporting membranes. At the same time, sporopodia projecting from the sporocyst surface towards the sporocyst veil and considered characteristic of *Calyptospora funduli* by Overstreet et al. (1984), were absent from the sporocysts of *G. sinensis*. Overstreet et al. (1984) distinguished two subgenera, *Goussia* and *Plagula*, within the genus *Goussia*. The difference between the subgenera is that the sporocysts of *Plagula* are surrounded by a sporocyst veil, while those of *Goussia* are not. Our studies have shown that this classification is unfounded: we observed a sporocyst veil also in *G. sinensis*, a typical member of the genus *Goussia* as evidenced by its sporocyst composed of two hemispheres connected by a suture. In our opinion the success or failure of demonstrating a sporocyst veil depends exclusively on the electron microscopic technique applied. It is highly probable that fish coccidia have a sporocyst veil. On the other hand, the sporopodia typical of *Calyptospora*

seem to be a real distinguishing character of that genus. Researchers of fish coccidia have long been intrigued by the origin of the so-called "yellow body" surrounding the oocysts. Molnár (1984) suggested that the yellow body was formed from the degenerated host cell and coagulated tissue fluid. The present studies seem to support, at least in part, the opinion of Kent and Hedrick (1985) who suggested that the yellow bodies were formed from degenerating membranes within secondary lysosomes and contained lipofuscin. At the same time, Figs 10 and 11 indicate that the origin of the "yellow bodies" is twofold: its granular part is constituted by the zygote residue not participating in oocyst formation, while the homogeneous outer part is probably formed by the necrotic cell or several aggregated cells. This may furnish an explanation for the finding that by light microscopy formations of granular structure are often seen together with the oocyst, or several oocysts, situated within the yellow bodies.

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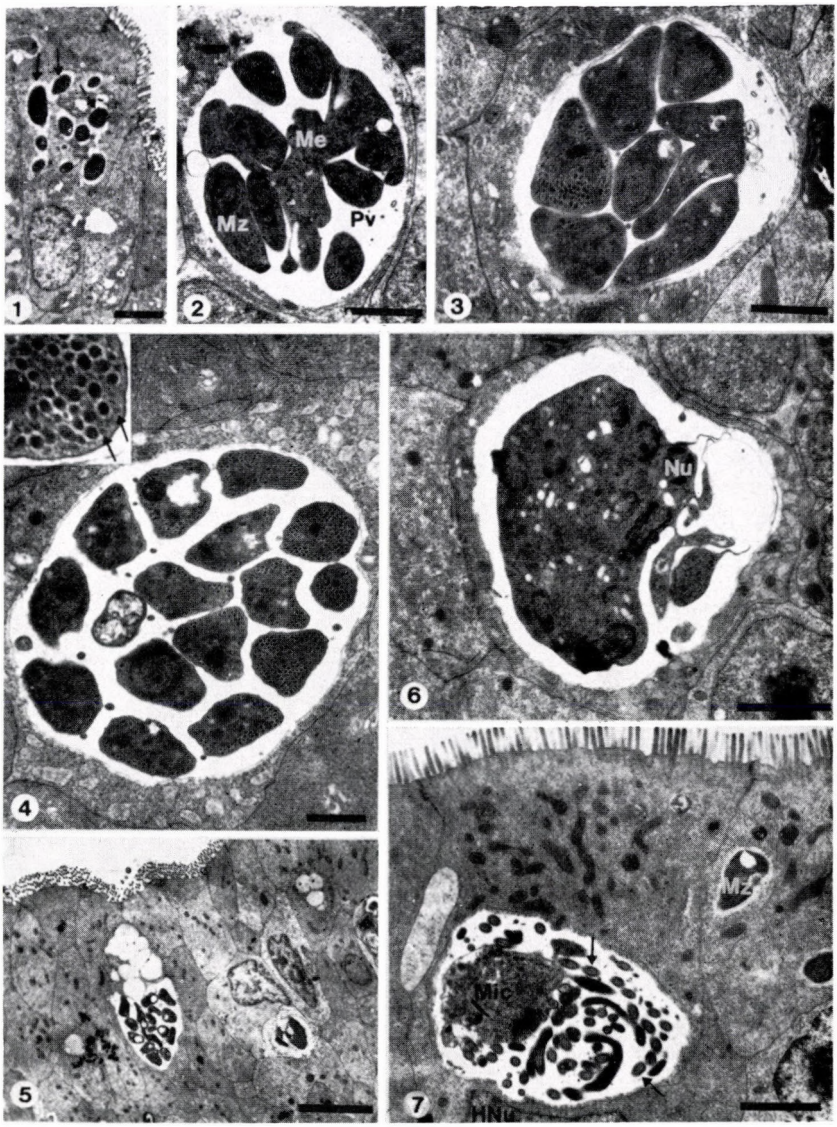

Fig. 1. Several merozoites (↓) in a single epithelial cell of the gut are seen apical to the nucleus. Bar = 2.5 μm

Fig. 2. Merozoites (Mz) have developed by strangulation from the meront (Me) situated in the parasitophorous vacuole (Pv). Bar = 2 μm

Fig. 3. A meront containing 8 merozoites within a gut epithelial cell. Bar = 1 μm

Fig. 4. Cross sections of merozoites (meront containing 16 merozoites). Insert: Note cross sections of subpellicular, longitudinally running tubules of a merozoite (↓). Bar = 1 μm

Fig. 5. Meront situated in the cytoplasm of a goblet cell, basal to the mucin droplets. Bar = 5 μm

Fig. 6. Young microgamont. Heterochromatic nuclei (Nu) are arranged peripherally in the cytoplasm of the microgamont. Bar = 2μm

Fig. 7. Cross sections of the perforatorium and flagellum of microgametes (↓) developing from a microgamont in a parasitophorous vacuole above the host cell nucleus (HNu). The other cell contains a merozoite (Mz). Bar = 2 μm

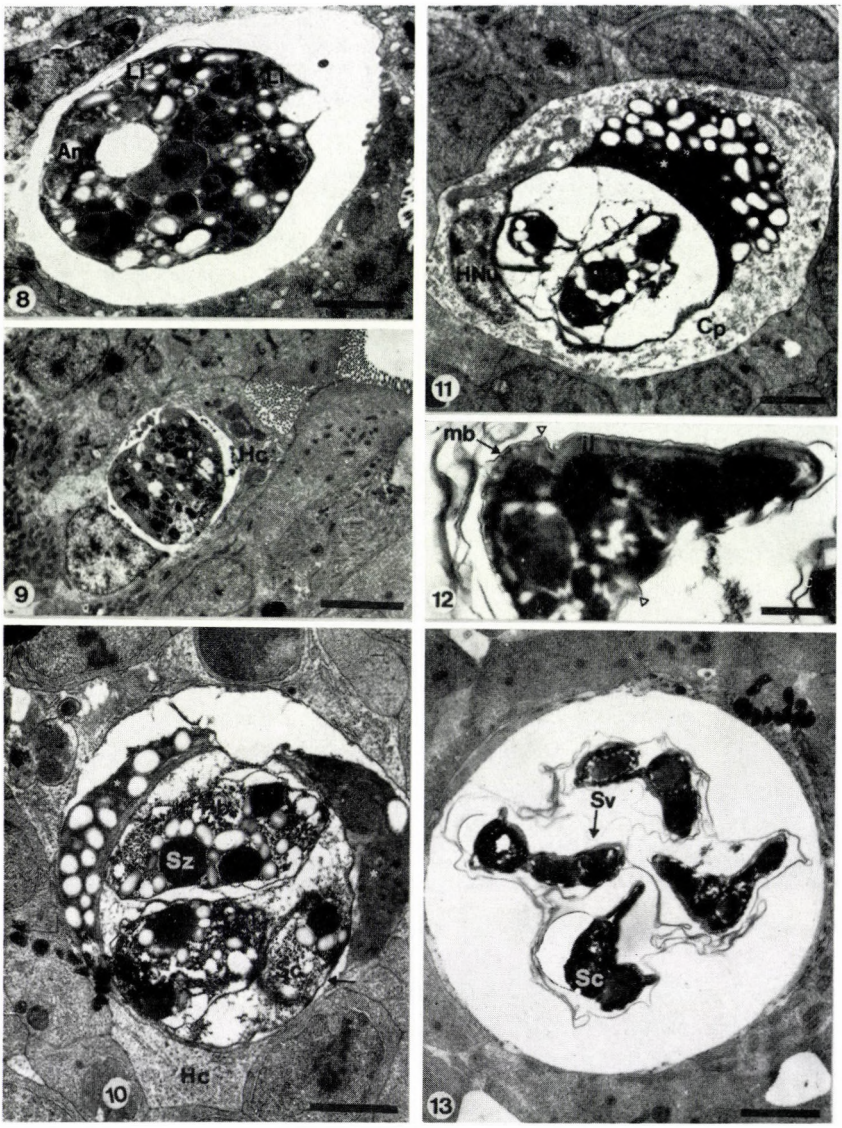

Fig. 8. Macrogamont. Note the amylopectin (Am) and lipid (Li) granules densely arranged in the cytoplasm. Bar = 2 μm

Fig. 9. The host cell carrying the enlarged parasite is pushed towards the deeper layers and its function is taken over by the neighbouring intact epithelial cells closing around the former. Bar = 5 μm

Fig. 10. Sporulating oocyst. Within the sporont almost completely filling the parasitophorous vacuole of the host cell (Hc) of disintegrated cytoplasm structure an oocyst surrounded by oocyst wall (↓) is developing. In cross sections of the sporocysts (Sc) note the sporocyst residual body (Rb) and primordia of the developing sporozoites (Sz). Note the outer residual body (*) attached to the oocyst wall from the outside. The residual body is constituted by that part of the sporont which has not participated in sporocyst formation. It has a highly electron-dense matrix, finely fibrillar arrangement, and contains nutrient granules. Bar = 2 μm

Fig. 11. Note sporulating oocyst in the cytoplasm (Cp), beside the nucleus (HNu) of a severely damaged, necrotic host cell. The extraoocystic residual body (*) is arranged asymmetrically and its substance contains nutrient granules identical in structure with that contained by the sporocyst residual body. Bar = 2 μm

Fig. 12. Cross section of a sporocyst. The sporocyst wall consists of an outer membrane (mb) and an inner layer (il) of homogeneous structure. The sporocyst wall consists of two hemispheres connected by a suture (△). Bar = 0.05 μm

Fig. 13. Sporulated oocyst. Each of the four sporocysts (Sc) is surrounded by a separate sporocyst veil (Sv). The oocyst wall is detached from the parasitophorous vacuole and lies on the sporocyst veil from the outside. Bar = 2 μm

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THE PATHOLOGY OF CONCURRENT BOVINE VIRAL DIARRHOEA AND INFECTIOUS BOVINE RHINOTRACHEITIS VIRUS INFECTION IN NEWBORN CALVES

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Concurrent bovine viral diarrhoea (BVD) and systemic infectious bovine rhinotracheitis (IBR) are reported from two neonatal (11 and 15 days old) calves. The diseases occurred sporadically in a large-scale herd which may have been due to the calves' heterogeneous immunobiological status. Gross pathological and histopathological examinations revealed focal interstitial pneumonia with acidophilic intranuclear inclusions in the alveolar epithelial cells and necrotic foci in the liver with a few intranuclear inclusions in the hepatocytes. There were subserous haemorrhages in the forestomachs and intestine, necrotic changes in the rumen, enteritis, lymphocytic necrosis in the Peyer's patches, and fibrinoid necrosis in the wall of some of the neighbouring blood vessels. BVD virus was demonstrated by immunofluorescence (IF), whereas IBR virus by electron microscopy, immunofluorescence and virus isolation.

Keywords: BVD, systemic IBR, neonatal calf.

In Hungary the occurrence of bovine viral diarrhoea (BVD) in neonatal calves was reported earlier (Romváry, 1965). The systemic form of infectious bovine rhinotracheitis (IBR) has also been reported from a suckled calf (Molnár et al., 1984). In the present paper pathological and diagnostic observations made on concurrent BVD and IBR in neonatal calves are reported. Simultaneous incidence of these two diseases in calves of that age has not been reported in the literature so far.

Materials and methods

Case history

The studies were performed in a cattle herd of an agricultural co-operative. The animals were kept in two dairy units. Calves from the farm's own calf crop were regularly mixed with pail-fed calves purchased from household and large-scale stocks. Simultaneously with the outbreak among neonatal calves, a combined BVD-IBR outbreak, diagnosed by clinical examination as well as virological and serological methods, took place among the pail-fed and weaned calves in both dairy units of the farm.

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In one of the units diarrhoea causing severe calf losses occurred among the neonatal calves which, depending on the available space, were kept tied down together with their dams for 10–15 days.

The outbreak lasted six weeks and was accompanied by almost 100% morbidity. Sixteen out of the 20 calves born to cows and 5 out of the 9 calves born to primiparous heifers died at 1–6 days of age. Three calf carcases were submitted to the laboratory: all three were diagnosed to have died of *Escherichia coli* diarrhoea caused by enteropathogenic K99 + *E. coli*.

Two calves that had suffered from diarrhoea during the first days of life but recovered as a result of therapy again became diarrhoeic at 8 and 10 days of age, respectively. The calves' body temperature was 41.2 and 41.5 °C, respectively; they were listless and did not suck. One day later they became dyspnoeic, were coughing, had serous nasal discharge and conjunctivitis. They did not respond to combined palliative treatment and antibiotherapy; both calves died 3 and 4 days later, respectively.

The carcases were submitted to the laboratory of the Central Veterinary Institute and necropsied.

Histopathological examination

Samples were taken from the lungs, trachea, liver, rumen wall, ileum, mesenteric lymph node, and brain (medulla oblongata, pons varolii, quadrigeminal bodies, hippocampus, cortex, and cerebellum), fixed in 5% (v/v) formalin (pH 7.2) buffered with NaH_2PO_4 and NaOH. Frozen or paraffin-embedded sections were prepared. For general information the sections were stained with haematoxylin and eosin. For demonstration of hyphae in the liver and rumen wall the periodic acid–Schiff (PAS) reaction was used.

Electron microscopy

Pieces of appropriate size were excised from the liver of one of the calves and fixed first in 2.5% (v/v) glutaraldehyde buffered with sodium cacodylate, then in 1% (v/v) osmium tetroxide. The samples were embedded in Durcupan resin, sectioned with an ultramicrotome, counterstained with uranyl acetate and lead citrate, and examined in an electron microscope.

Bacteriological examination

Samples taken from the spleen, liver, kidneys and lungs were tested on blood agar and Drigalski's agar plates and those taken from the small intestine and mesenteric lymph nodes on common agar, Drigalski's and Minca's agar plates. For bacteriological examination of the liver and rumen wall samples

Sabouraud agar was also used. The cultures were incubated at 37 °C. Smears prepared from the small intestinal contents were stained by Castaneda's method.

Virological examinations

The immunofluorescence test (IF) was used and virus isolation was attempted. The smears made from the tonsils, lungs, spleen, liver, small intestinal mucosa and mesenteric lymph node on slides were fixed in a 3 : 1 mixture of acetone and methanol at room temperature for 20 min. For the demonstration of IBR virus antigen direct, while for BVD virus antigen indirect IF was used as described previously (Pálfi and Molnár, 1987). Virus isolation was attempted from homogenates prepared from the above-listed organs in secondary calf testicle cell cultures. The isolates were identified by IF. If virus isolation appeared negative, three blind passages were performed.

Serological tests

Blood samples were taken from 10 pregnant cows 2 to 4 weeks before term. The samples were assayed for the presence of BVD and IBR antibodies by the virus neutralization (VN) test.

Results

Gross pathology

Two calves that had died 11 and 15 days old, respectively, were examined.

At necropsy similar lesions were found in both calves. The mucous membrane of the nasal cavity and epiglottis was dark red and swollen, that of the trachea was also red and in its anterior third there were circumscribed, superficial necroses. The lungs were more compact to the touch than usual, and in the cardiac and apical lobes as well as in the anterior third of the diaphragmatic lobes there were compact, greyish-red, atelectatic areas variable in size. The liver was enlarged, yellowish-brown or reddish, its substance was more easy to tear, and on its surface and cut surface there were numerous yellowish foci the size of a pinhead (Fig. 1). In the renal cortex a few punctate haemorrhages, whereas under the capsule of the spleen extensive haemorrhages were seen. Under the serosa of the forestomachs there were extensive haemorrhages and on the mucous membrane of the rumen superficially necrotic pseudomembranes the size of an egg or half palm were seen. The pseudomembranes were easily detachable from their base, which was reddened

(Fig. 2). The mucous membrane of the abomasum and intestinal tract was reddened, swollen and contained a few punctate haemorrhages. In the small intestine the Peyer's patches were swollen, reddened and above them the mucous membrane was, here and there, necrotic. Under the serosa of the intestinal wall there were punctate, in some places "sprinkled", haemorrhages (Fig. 3). The mesenteric lymph nodes were swollen, succulent and their cortex was reddened.

Histopathological and electron microscopic studies

By *light microscopy*, in the *lungs*, in the interalveolar tissues and wall of the major air passages (in areas exhibiting gross lesions) infiltrations consisting of lymphocytes, histiocytes, plasma cells and a few neutrophilic granulocytes were seen. In these areas there was circumscribed cell necrosis with karyopyknosis or karyorrhexis (Fig. 4). Some alveolar epithelial cells had detached and formed, here and there, syncytium-like giant cells, some of which contained intranuclear inclusion bodies. In the mucous membrane of the *trachea* there were subepithelial infiltrations of lymphocytes and histiocytes, and minor haemorrhages. In circumscribed areas of the *liver*, necrosis of the parenchymal cells was observed (Fig. 5). Necrosis occurred in different parts of the lobules and usually extended to one-fourth to one-third of the lobule. In these foci nuclear residues and outlines of the more or less intact cells of the RHS were recognizable. In the marginal parts of the necrotic areas some of the chromatin substance of the enlarged hepatocyte nuclei was attached to the nuclear membrane in clumps and showed intense basophilic staining (perichromasia), while the majority of chromatin completely filled the nucleus and showed bright eosinophilic staining (Fig. 6). Occasionally the acidophilic area seen in the enlarged nucleus was surrounded by a lighter zone (Cowdry type A intranuclear inclusion). Here and there the necrotic solitary hepatocytes had been transformed into a bright eosinophilic, disk-like body (Councilman body). In the liver the diffuse, exudative lesions were less pronounced. Here and there the perivascular lymph spaces and the sinusoids of lobules were dilated and in their lumen a serous exudate containing a few lymphocytes and granulocytes had accumulated. The endothelial cells of the sinusoids and the Kupffer's cells were slightly swollen and occasionally detached. In other places proliferation of cells of the RHS was observed. The interlobular septa were in some places slightly infiltrated by lymphocytes and histiocytes.

By *electron microscopy of the liver* viral particles were only seen in the necrotic areas. In these areas the cell organelles of the hepatocytes were mostly unrecognizable and the cell membrane as well as the nuclear membrane had undergone lysis. Enveloped viral particles of icosahedral symmetry and

about 150 nm in diameter were seen singly, diffusely or in groups. The particles represented different stages of maturation (Fig. 7). At the periphery of the lesions the nuclei of hepatocytes had become enlarged and in some places the perinuclear space was dilated. Part of the chromatin substance congregated along the nuclear membrane, while the remaining part accumulated in the centre of the nucleus as granular or filamentous material. No viral particles were seen in these cells.

Light microscopy of the rumen wall revealed, in some portions of the mucosal epithelium, vacuolar degeneration and, here and there, necrosis extending to the submucosa. The submucosa was hyperaemic and infiltrated by inflammatory cells (lymphocytes, histiocytes and neutrophilic granulocytes). In the wall of some blood vessels the endothelial cells were degenerated and the tunica media showed fibrinoid necrosis (Fig. 8). Above the Peyer's patches the epithelium of the *ileal mucosa* was necrotic and the lamina propria showed serous, haemorrhagic, fibrinous infiltration and was infiltrated by inflammatory cells. The majority of lymphocytes had been depleted from the lymphoid follicles and those remaining there showed signs of necrosis (pyknosis, rhexis; Fig. 9). The wall of some blood vessels exhibited fibrinoid necrosis. In the sinusoids of the *mesenteric lymph nodes* accumulation of serofibrinous exudate, haemorrhages, swelling of macrophages, and proliferation of neutrophilic granulocytes were seen, while in the lymphoid follicles of the cortex there was lymphocyte depletion. In the blood vessels of the *brain* the endothelial cells were slightly swollen. In the lumen of these blood vessels leucocytes had increased in number and in the perivascular areas here and there haemorrhages were seen. No mycelia were demonstrable by the PAS reaction.

Bacteriological examinations. We failed to culture bacteria from the parenchymal organs. The aerobic microflora of the small intestine consisted of enterococci and non-mucoid type *Escherichia coli*. Attempts at demonstrating fungi from the liver and rumen wall of the calves consistently failed.

Virological examinations. By the immunofluorescence (IF) test IBRV antigen was demonstrated in the tonsils, lungs, spleen and liver, whereas BVDV antigen in the small intestine and mesenteric lymph nodes of both calves. Virus isolation attempts from the tonsils, lungs and livers yielded IBRV. In cultures inoculated with homogenates of the small intestines and mesenteric lymph nodes no cytopathic effect was observed during three passages. After the third passage the cell cultures were tested by IF for the presence of noncytopathic BVDV. The tests were consistently negative.

Serological tests. Six and 9 out of the 10 pregnant cows were shown to possess virus-neutralizing antibodies to BVDV and IBRV, respectively.

Discussion

Numerous papers have been published on diseases caused in neonatal calves by BVDV and IBRV. At that age BVDV produces a disease characterized by fever, leucopenia, nasal discharge, diarrhoea and, in some cases, erosions on the mucous membrane lining the digestive tract (Lambert, 1974; Romváry, 1965). IBR has various manifestations in neonatal calves, characterized by involvement of the nervous system (Lomba et al., 1974), digestive tract (Wellemans et al., 1974), and respiratory tract (Lomba et al., 1973). Its systemic form (Reed et al., 1973) has also been described.

Concurrent BVDV and IBRV infection has so far been observed among bucket-reared, weaned and growing calves (Eugster et al., 1976; Greig et al., 1981; Pálfi and Molnár, 1987). In these cases digestive symptoms typical of BVD were consistently accompanied by the respiratory form of IBR.

The interesting feature of the case presented here was that concurrent BVD and IBR occurred in neonatal calves and that BVD was accompanied by systemic (generalized) IBR characterized by hepatic lesions. It is remarkable that at that age the disease occurred sporadically, despite the fact that all conditions favouring its spread were present. This was indicated by a simultaneous outbreak of *Escherichia coli* diarrhoea and the concurrent BVD-IBR outbreak occurring among bucket-reared and weaned calves housed in other buildings of the same farm. A similarly sporadic occurrence of systemic IBR among neonatal calves was reported earlier (Molnár et al., 1984). As it was suggested by us earlier, the sporadic nature of the present outbreak must have been due to heterogeneity in the immune status of the suckler calves, i.e. to the lack of colostrum-derived immunity in some animals. This is supported by the serological results obtained in cows close to calving: 6 and 9 out of 10 serum samples contained antibodies to BVDV and IBRV, respectively.

The clinical symptoms were atypical. The gross pathological lesions, however, were suggestive of concurrent BVDV and IBRV infection. Lesions of the upper respiratory tract, lungs, and primarily those of the liver were indicative of IBRV, whereas the digestive lesions and haemorrhages were suggestive of BVDV infection. Lesions similar to those found in the mucous membrane of the forestomachs are often found in association with mycotoxicoses (Dankó, 1972). In the present case, however, the bacteriological and histopathological findings ruled out this possibility.

In addition to the demonstration of BVDV by IF and that of IBRV by IF, electron microscopy and virus isolation, the histopathological findings offered evidence of concurrent BVDV and IBRV infection. Ulceration of the mucous membrane of the forestomachs and small intestine, accompanied by vacuolar degeneration and necrosis of the epithelium and lymphocyte depletion and necrosis in the Peyer's patches, as well as degeneration and fibrinoid

necrosis of the blood vessel walls in that region were indicative of BVDV action (Baker et al., 1954; Carlson et al., 1957; Kendrick, 1971; Lambert et al., 1969; Jones and Hunt, 1983). On the other hand, intralobular interstitial pneumonia, necrotic foci and intranuclear inclusions in the lungs, necrotic foci and presence of herpesvirus particles among the necrotic cell debris in the liver indicated the development of systemic IBR (Ehrensperger and Pohlenz, 1979; Miller et al., 1973; Molnár et al., 1984).

The unusually high mortality (21 out of 29 neonatal calves died) observed during the outbreak of *E. coli* diarrhoea suggested that BVDV was present in several of these calves as concomitant infection. Investigations into this aspect were not possible in the present case. In the future, however, in similar cases the laboratory examinations will be extended to checking the presence of BVDV as well.

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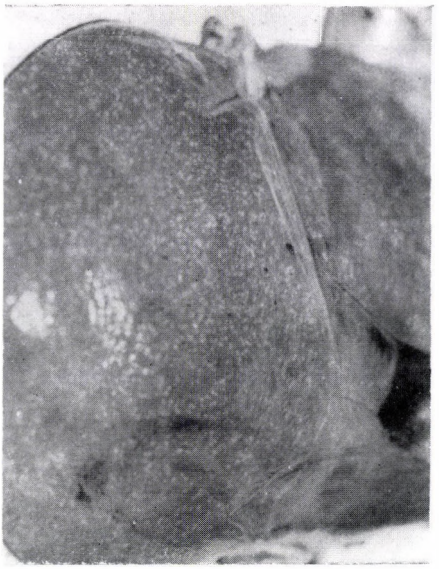


Fig. 1. Necrotic foci in the liver

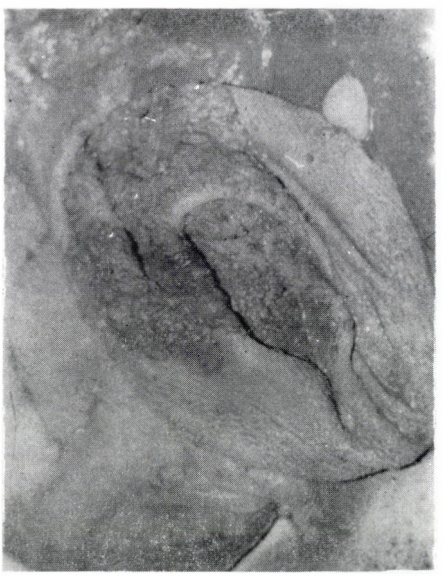


Fig. 2. Extensive necrosis of irregular shape in the rumen mucosa

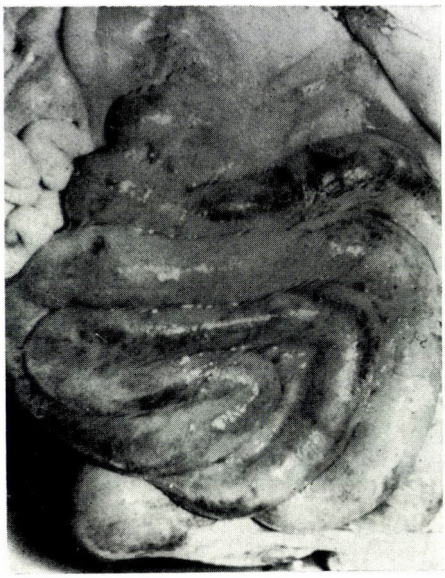


Fig. 3. Punctate, in some places "sprinkled", haemorrhages under the serosa of the intestine

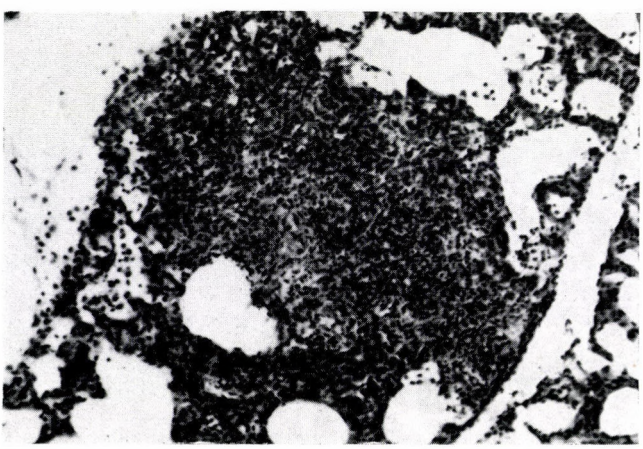


Fig. 4. Inflammatory-necrotic focus in the widened interalveolar tissue of the lungs. Haematoxylin and eosin (HE), $\times 160$

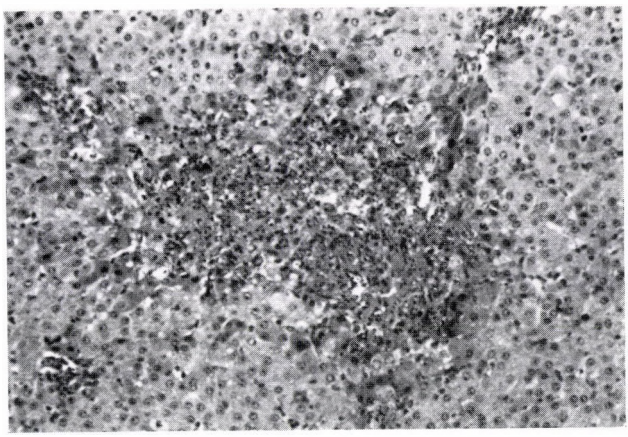


Fig. 5. Necrotic focus in the liver. HE, ×63

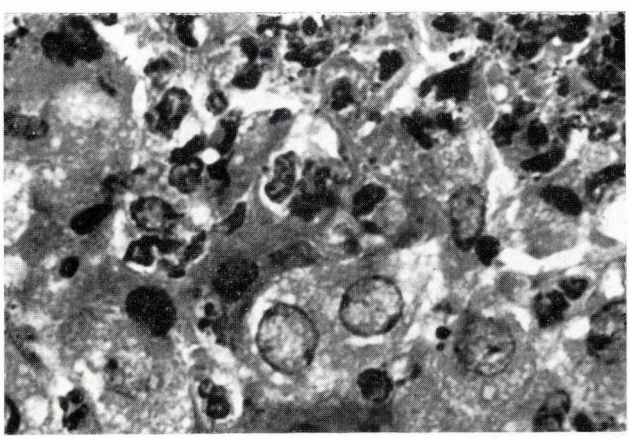


Fig. 6. Detail of Fig. 5. HE, ×400

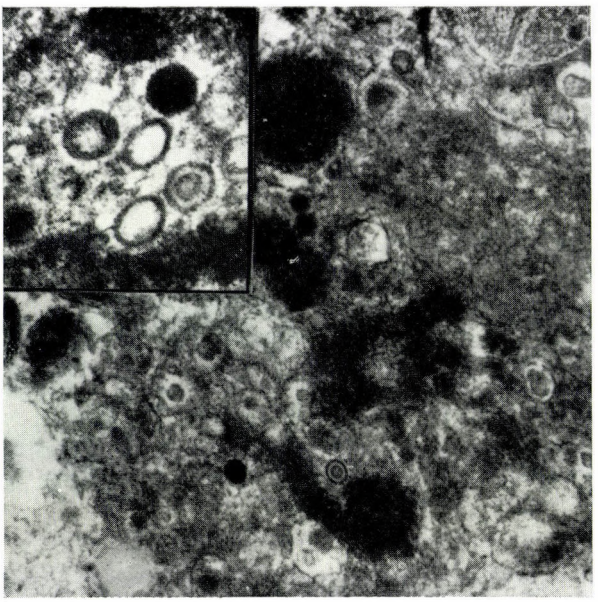


Fig. 7. Viral particles representing various stages of maturation in the necrotic area of the liver. Electron micrograph, ×44,500. Top left: ×70,000

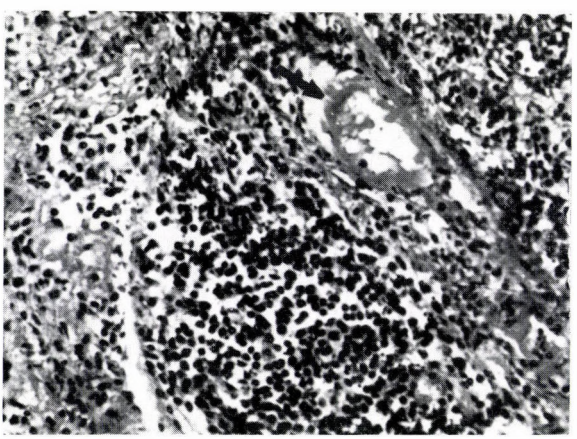


Fig. 8. Fibrinoid necrosis of the blood vessel wall (arrow) in the rumen wall infiltrated by inflammatory cells. HE, ×160

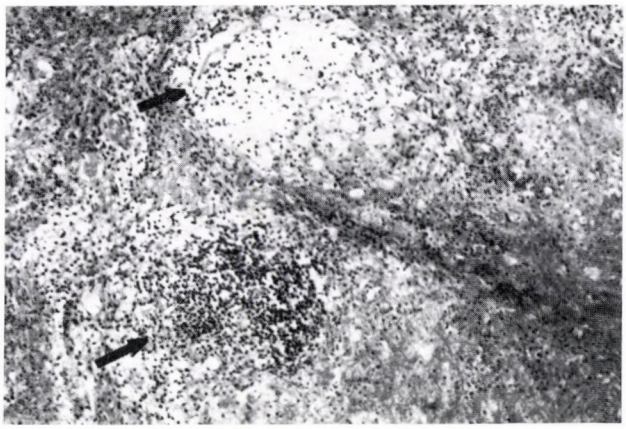


Fig. 9. Lymphocyte depletion in the lymphoid follicles (arrow) and necrosis in the wall of the ileum. HE, ×63

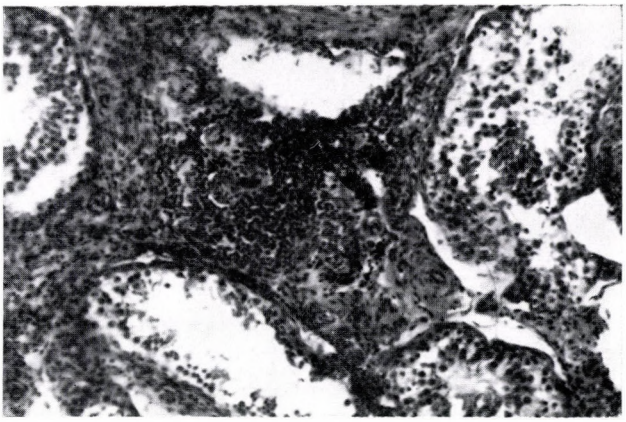
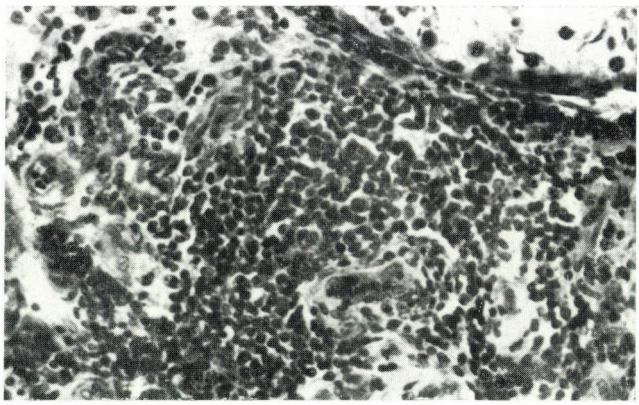
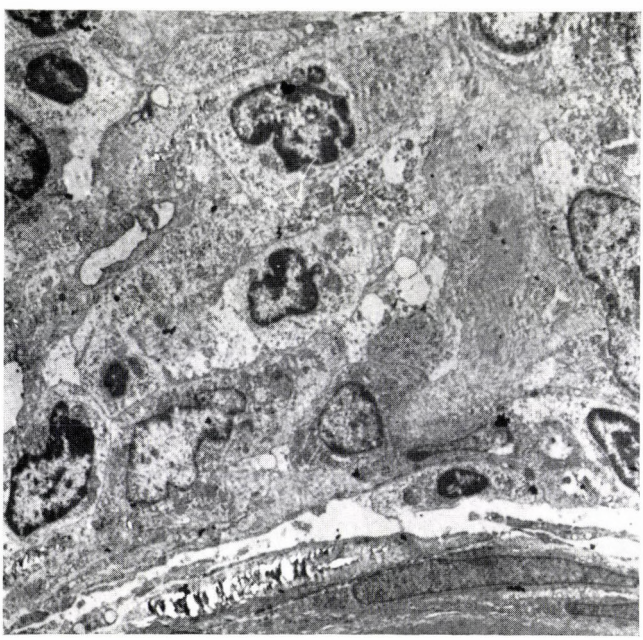


Fig. 1. Infiltration of the interstitium of testicle with mononuclear cells. Note incipient atrophy of the neighbouring seminiferous tubules. Haematoxylin–eosin (H–E.), ×63





Figs 2 and 3. Among the lymphocytes and histiocytes in some places there are fibroblasts. H–E., ×160 (Fig. 2) and electron micrograph, ×4100 (Fig. 3)

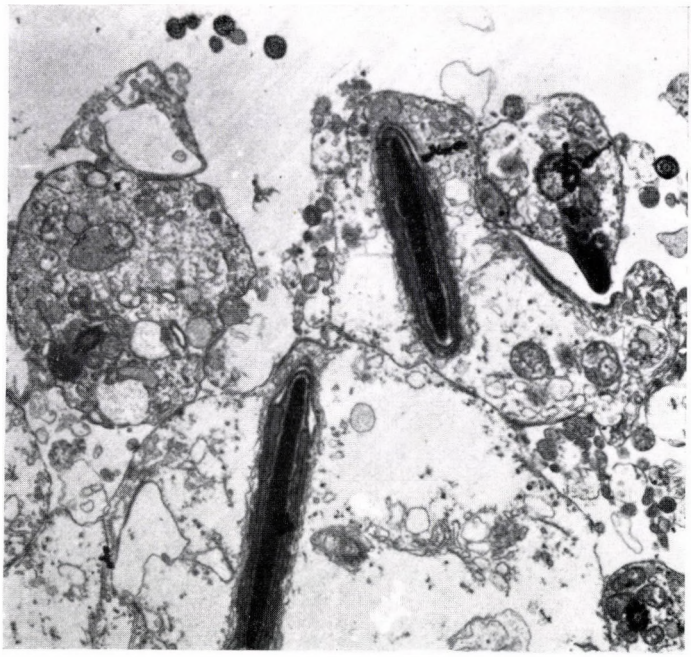


Fig. 4. Note some spermatozoa among the detached and degenerated remnants of germinal epithelial cells. Electron micrograph, $\times 9700$

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TESTICULAR LESIONS IN RAMS INFECTED BY MAEDI/VISNA VIRUS

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Pathological lesions were observed in the testicles of 5 out of 7 rams found to be infected by maedi virus serologically and by histopathological examination of the lungs. The interstitium of the testicles was infiltrated with lymphocytes, histiocytes and plasma cells. The infiltration was mainly perivascular and of varying severity, and was accompanied by fibrosis. The seminiferous tubules neighbouring the severely affected parts were atrophied and, as a result, in circumscribed areas of the testicles disturbances of spermatogenesis were observed. The epididymides were devoid of pathological changes. Studies are in progress to determine whether the testicuar lesions are actually caused by maedi/visna virus. Transmission of the virus with infected rams' semen is possible.

Keywords: Maedi/visna, testicular lesions, ram.

Maedi/visna is a lentiviral disease of sheep which may appear as progressive interstitial pneumonia (maedi) or as meningo-leukoencephalitis (visna), depending on the predominant clinical symptoms. Maedi/visna characterized by chronic respiratory symptoms was described in Hungary in 1973 (Süveges and Széky, 1973). In addition to changes in the lungs and peribronchial lymph nodes, lymphocytic encephalitis was observed in one animal. In animals infected by maedi/visna virus other authors observed chronic indurative mastitis (Griem and Weinhold, 1976; Oliver et al., 1981; van der Molen and Houwers, 1987) and chronic nonpurulent arthritis (Cutlip et al., 1985*a*; Narayan and Cork, 1985). Cutlip et al. (1985*b*) demonstrated that the virus induced inflammation of the arterioles running in the articular capsule, kidneys, brain and meninges, lungs and tracheal wall. Griem and Weinhold (1976) reported that perivascular focal infiltration with mononuclear cells might occur all over the body. Infected rams are assumed to play a role in transmitting infection (Süveges and Széky, 1973). However, no testicular lesions of infected rams have been reported so far. In this paper the pathological lesions found in the testicles of rams infected by maedi/visna virus are described.

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Materials and methods

Animals

Nine 5 to 8 years old rams slaughtered at the local abattoir were examined by different methods. The rams had been culled from a large flock where serological and pathological examination of the ewes revealed severe maedi/visna infection.

Blood samples

Blood samples were collected in sterile tubes. The sera were separated, inactivated at 56 °C for 30 min, and stored frozen until used.

Serology

To demonstrate antibodies to maedi/visna virus, sera were tested by agar-gel immunodiffusion (AGID), whereas for infection by Border disease virus they were examined by virus neutralization (VN).

AGID test

0.7% agar (Noble, Difco) prepared in borate buffer, pH 8.6, was used. The antigen and the positive serum were a kind gift from Dr. R. C. Cutlip (Ames, Iowa, U.S.A.).

VN test

1 : 5 dilutions of the sera were tested against 100 $TCID_{50}$/0.1 ml of BVD strain $C_{24}V$ Oregon in calf testicle cell cultures.

Histopathology and electron microscopy

For histopathology, samples were taken at necropsy from the lungs, peribronchial lymph nodes and both testicles and epididymides. The samples were fixed in 5% (v/v) formalin (pH 7.2), buffered with NaH_2PO_4 and NaOH, and frozen as well as paraffin-embedded sections were prepared. Fettrot staining was used to demonstrate Leydig's interstitial cells. One testicle each of animals 1 and 2 was examined by electron microscopy. Pieces were fixed in 2.5% (v/v) glutaraldehyde buffered with sodium cacodylate, then post-fixed in 1% (v/v) osmium tetroxide. The samples were embedded in Durcupan resin, semi-thin sections were cut with an ultramicrotome, stained with toluidin blue and examined with a light microscope. Ultrathin sections were counterstained with uranyl acetate and lead citrate and examined with an electron microscope.

Results

The results of serological and histopathological examinations are summarized in Table I.

Table I

Results of the AGID test and histopathological findings

No. of animal	AGID test	Histopathological lesions	
		Lungs	Testicles
1	+	+	+
2	+	+	+
3	+	+	+
4	+	+	+
5	+	+	+
6	+	+	−
7	+	+	−
8	−	−	−
9	−	−	−

Serology

Precipitating antibodies to maedi/visna virus were demonstrated in the sera of 7 sheep. None of the sheep were found to possess antibodies to BVD virus.

Histopathology and electron microscopy

In sheep 1 to 7 chronic interstitial pneumonia considered typical of maedi and characterized by formation of lymphoid follicles, fibrosis, and hypertrophy of the smooth muscle elements was observed. In these animals in the cortex and paracortical zone of peribronchial lymph nodes there was lymphoid hyperplasia, whereas in the lumen of sinusoids proliferation of macrophages was seen.

In the testicles of rams 1, 2 and 3 there was focal, and here and there diffuse, infiltration with lymphocytes, lymphoblasts, histiocytes and plasma cells in the loose connective tissue between the seminiferous tubules, mainly around blood vessels (Figs 1, 2 and 3). In the larger infiltrated areas there was atrophy of Leydig's interstitial cells and proliferation of fibroblasts. In the neighbouring seminiferous tubules atrophy and degeneration of the germinal epithelium were seen (Figs 1 and 4). No viral particles were observed in the altered areas.

No pathological lesions were seen in the epididymis.

In the testicles of rams 4 and 5 the infiltrations were fewer in number and less marked. Neither degenerative lesions nor fibrosis was observed.

Discussion

Of genital infections of rams, those of bacterial origin are well known (Jansen, 1980*a*; Meyer, 1982; Burgess, 1982; Walker et al., 1986), while only few data have been published on changes produced by viruses (Watt, 1972).

Bacterial infections of the internal genital organs manifest themselves either as clinically apparent epididymo-orchitis or as subclinical disease accompanied by inflammation of the accessory sex glands (Baynes and Simmons, 1968; Watt, 1978; Jansen, 1980*a*, *b*; Burgess, 1982). In the aetiology of these diseases three Gram-negative bacteria, *viz. Brucella ovis*, *Histophilus ovis* and *Actinobacillus seminis*, are the most important (Meyer, 1982; Burgess, 1982).

According to the present knowledge, the aetiology of bacterial infections of the internal genital organs is usually different in ram hoggets and in breeding rams (Walker et al., 1986). In ram hoggets acute or subacute purulent epididymitis and/or orchitis, as well as chronic epididymitis, are most often caused by *H. ovis* and *A. seminis* strains (Van Tonder, 1973; Webb, 1983; Walker et al., 1986). On the other hand, in breeding rams the most important cause of genital infection is *B. ovis* all over the world (Watt, 1972; Meyer, 1982; Burgess, 1982). Under natural conditions, in typical cases *B. ovis* causes chronic unilateral or bilateral epididymitis mainly restricted to the cauda epididymidis and accompanied by spermatocele formation (Jubb and Kennedy, 1970; Meyer, 1982; Burgess, 1982). In the testicles usually only secondary regressive lesions (e.g. atrophy, calcification) can be observed (Watt, 1972; Burgess, 1982).

Virus-induced testicular lesions have been reported in connection with sheep pox (Jubb and Kennedy, 1970), lumpy skin disease (Nieberle and Cohrs, 1967), and Border disease (Barlow and Gardiner, 1983). The virus may persist for months or years in the seminiferous tubules of ram hoggets affected by Border disease but reaching maturity (Terpstra, 1978, 1981.) These rams are of impaired fertility due to testicular hypoplasia (Barlow and Gardiner, 1983), excrete the virus with their semen (Gardiner and Barlow, 1981), and may transmit infection to the ewes during mating (Terpstra, 1981; Barlow and Gardiner, 1983). Border disease has recently been shown to occur in Hungary (Hajtós et al., 1988). In the case presented here the negative results of serological tests and on-the-spot clinical examinations allowed us to rule out the involvement of Border disease virus. Sheep pox and lumpy skin disease were excluded on the understanding that these diseases do not occur in Hungary. Epididymo-orchitis of bacterial origin could be ruled out since in the rams infected by maedi/visna virus histopathological lesions of the testicles were not accompanied by simultaneous damage of the epididymides.

In 7 out of the 9 rams examined the serological results as well as the histopathological lesions found in the lungs proved that the rams were infected by maedi/visna virus. Five of these rams exhibited testicular lesions,

primarily proliferative perivascular infiltration with lymphocytes, lymphoblasts, histiocytes and plasma cells, accompanied by fibrosis and circumscribed atrophy of the seminiferous tubules. Similar pathological processes may develop in the mammary glands, articular walls and other organs of animals infected by maedi/visna virus, in addition to lesions occurring in the lungs (Jones and Hunt, 1983; Dawson, 1987). From the results it seems obvious that the lesions observed in the testicles can be attributed to maedi/visna virus. Although the lesions were observed in the interstitium of the testicles, the more extensive lesions resulted in atrophy of the seminiferous tubules and, thus, local disturbances of spermatogenesis. Damage of Leydig's interstitial cells may, in our assumption, lead to disorders in the secretion of sex hormones.

Maedi/visna virus spreads horizontally within flock (De Boer, 1970). The most important mode of spread is via the colostrum and milk (De Boer et al., 1979), but the virus may also be transmitted with respiratory secretions and saliva (Straub, 1970; Narayan and Cork, 1985). Epizootiological data indicated the role of rams in transmitting infection (Süveges and Széky, 1973) but no data have been published on the mode of spread. The testicular lesions found in rams infected by maedi/visna virus suggest that the virus can be transmitted, besides the respiratory secretions and saliva, with semen. Thus, at mating an infected ram may infect large numbers of susceptible ewes. This is supported by lower productivity due to reduced conception rate observed in ewe flocks subclinically infected with maedi/visna virus (Dohoo et al., 1987).

Infection experiments aimed at confirming the role of maedi/visna virus in producing testicular lesions are currently under way. The results will be reported in another paper.

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IN VITRO EVALUATION OF PROTEIN DEGRADABILITY IN THE RUMEN AND DIGESTIBILITY OF UNDEGRADED PROTEIN

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A three-phase laboratory procedure suitable for predicting protein degradability in the rumen and digestibility of undegraded protein is reported. In the first phase the feed was incubated with starch and buffered rumen fluid. In the incubation mixture the viability of protease-active bacteria was checked by anaerobic culturing, whereas changes in protease activity were monitored by azocasein degradation. In the second and third phase rumen *und*egradable *p*rotein (UDP) was digested with pepsin and pancreatin, respectively. The measurements showed that 63.2, 5.2 and 4.7% of the crude protein of green lucerne was decomposed by rumen fluid, pepsin and pancreatin, respectively. Degradability of the crude protein of extracted sunflower meal was 68.3, 17.7 and 5.5% in the three phases, respectively. Repeated determination yielded crude protein degradabilities of 66.7, 27.1 and 5.1% for the three phases, respectively.

Keywords: Protein degradability, digestibility of rumen undegraded protein, protein evaluation for ruminants, *in vitro* investigations.

In ruminants proteolysis mainly takes place at three sites of the digestive tract, *viz.* in the rumen, abomasum, and small intestine. In contrast to postruminal digestion, metabolic processes in the rumen are characterized not only proteolysis but also by very substantial protein synthesis. As a result of the proteolytic and protein-synthesizing activity of symbiont microorganisms the protein leaving the rumen differs from that entering it both in quantity and quality. Only protein rendered suitable for absorption from the small intestine during postruminal digestion can contribute to meeting the host animal's protein requirements. This may come from the following sources: (1) bacterial and protozoan proteins; (2) rumen undegradable protein (UDP), i.e. that portion of feed protein which passes the rumen undegraded; (3) endogenous proteins (e.g. digestive enzymes, desquamated cells).

Varying proportions of the different feed proteins are degraded in the rumen (Hume, 1974; Ørskov, 1982; Tamminga, 1983) and the amount of protein synthesized in the rumen can be substantial. As much as 50 to 90% of the protein N reaching the duodenum can be of microbial origin (Miller et al., 1982). Because of the above processes taking place in the rumen, in rumi-

nants evaluation of feed protein content is much more complicated than in monogastric animals. Although researchers have elaborated numerous procedures for determining the degradability of different proteins, no wholly satisfactory procedure is yet available. Several critical analyses of the advantages and drawbacks of these procedures have been published in the literature (Miller et al., 1982; Ørskov, 1982; Stern and Satter, 1984; Veresegyházy and Fekete 1987). Procedures utilizing fresh rumen fluid in an *in vitro* system seem to be suitable for wide use and routine testing because they are rather simple and easy to standardize (Tilley and Terry, 1963; Akeson and Stahman, 1964; Dougherty and Church, 1982; Merry et al., 1983; Czerkawsky and Breckenridge, 1985; Broderick, 1987; Madsen and Hvelplund, 1985).

This paper reports an *in vitro* system which seems suitable for predicting feed protein degradability in the rumen and digestibility of rumen bypass protein. The method is demonstrated on testing the protein degradability of a protein-rich roughage (green lucerne) and a fodder (extracted sunflower meal). Our choice fell on feeds which have a major role in the protein supply of ruminants, have markedly different crude protein content, and on which numerous data are available in the literature.

Materials and methods

Principle of the method

The three-phase laboratory method reported in this paper was used to simulate protein digestion taking place in three different portions of the ruminant digestive tract.

During the *first phase* the proteolytic and protein-synthesizing activities of ruminal microorganisms are estimated. Using the method of Tilley and Terry (1963), small quantities of finely ground feed are incubated with fresh, filtered rumen contents under anaerobic conditions for 48 h. To prevent energy deficiency from limiting the rate of bacterial protein synthesis, starch is added to each incubation mixture. Each test includes two phase-one incubations. One serves for determining, after an incubation of 48 h, the quantity of insoluble crude protein, whereas the sediment obtained by centrifuging the second incubation mixture is used as starting material for the second phase. Namely, this sediment contains the undegraded portion of the feed (including rumen bypass protein) and the microorganisms.

The *second phase* serves for estimating the expectable degree of abomasal protein degradation by digesting the sediment in freshly prepared pepsin-HCl solution for 48 h. The quantity of the digested protein is determined from the supernatant obtained by centrifugation.

In the *third phase* the sediment is digested further with pancreatin. The degree of small-intestinal protein digestion is indicated by the quantity of protein dissolved as a result of pancreatin digestion.

The principle of the method is shown in Fig. 1. To measure protein quantity in the filtered ruminal fluid and that originating from bacterial proliferation independent of the feed, a blind test is performed.

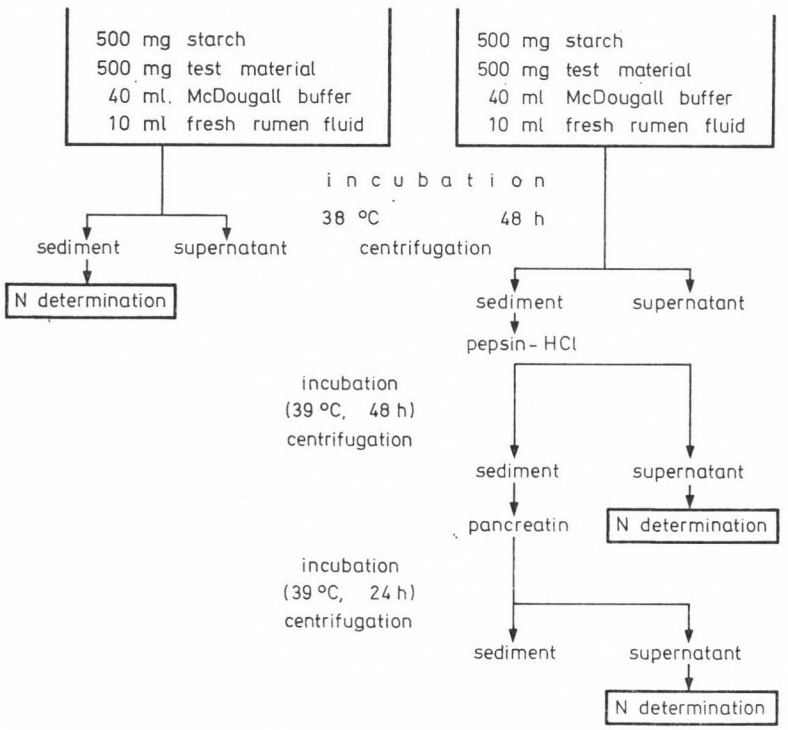

Fig. 1. Block diagram of the three-phase *in vitro* method simulating protein digestion in ruminants

Description of the method

Material. Green lucerne in flowering (crude protein content: 174 g/kg dry matter) and extracted sunflower meal (crude protein content: 420 g/kg dry matter) were used. Prior to digestion, green lucerne was dried cautiously at 45 °C during ventilation for several days to avoid protein denaturation. Drying was continued until air-dryness, then the samples were ground finely. This material was used for the *in vitro* determinations. Extracted sunflower meal was sifted through a sieve of two different pore sizes so that the particles used for the measurements were between 1 and 2 mm in diameter.

Rumen contents were obtained from a Worsted Merino wether of about 45 kg body mass and fitted up with a chronic rumen fistula. The daily ration of this animal comprised 0.25 kg extracted sunflower meal, 0.5 kg lucerne hay of medium quality, 1.0 kg corn grits, and calcium/phosphorus supplements (Phylaphor and mineral premix III). The daily ration was fed in two equal parts at 8 o'clock a.m. and 3 o'clock p.m. Licking-salt and drinking water were available *ad libitum*.

Prior to use the rumen contents were filtered through a 3-layered gauze twice. Thirty to forty min after collection the rumen fluid was under anaerobic conditions again.

The rumen fluid was buffered with the "artificial saliva" described by McDougall (1948).

As energy source water-soluble starch (REANAL Fine Chemicals Co., Budapest, cat. no. 11125-2-30-38) was used.

The pepsin solution was prepared from crystalline pepsin of pharmaceutical quality according to specifications of the A. O. A. C. (1975), 30 to 60 min before use.

The pancreatin solution was prepared by dissolving Cotazym forte tablets (EGIS Pharmacochemical Works, Budapest). Each tablet contained 260 mg pancreatin A and 15 mg cellulase enzyme. For each test 1 pulverized Cotazym forte tablet was dissolved in 20 ml 0.2 mol/l phosphate buffer (pH 8.0).

First phase. To each of two 100-ml thick-walled glass centrifuge tubes, 500 mg starch and 500 mg feed sample were added. Subsequently, 40 ml McDougall's buffer and 10 ml filtered rumen fluid were pipetted onto the samples. CO_2 was bubbled through the reaction mixtures for 2 min, then the centrifuge tubes were quickly sealed with rubber stoppers. The rubber stoppers were previously fitted up with a simple valve described by Tilley and Terry (1963).

The tubes were placed in a water-bath of 38 °C for 48 h. During incubation the samples were shaken up by hand at 6- to 8-h intervals. At the end of incubation bacterial metabolism was stopped by adding 2 ml $HgCl_2$ of 73.6 mmol/l concentration. The mixtures were centrifuged in a 7-cm rotor of a Janetzki T 24 centrifuge at 10,000 rpm for 10 min and the supernatants were discarded. One of the two sediments was stored at —20 °C until used for crude protein determination, whereas the second was digested further during the second phase.

Second phase. Onto one of the sediments obtained during the first phase a freshly prepared pepsin-HCl solution was pipetted. Digestion with pepsin was performed as described by the A. O. A. C. (1975) with the difference that we calculated with 0.5 g instead of 2 g of sample. After the 48th h the mixtures were centrifuged as described for the first phase. The supernatant was stored at —20 °C until used for crude protein determination.

Third phase. Onto the sediment obtained in the second phase 20 ml pancreatin solution was measured, and the mixture was incubated at 39 °C for 24 h. After centrifugation the supernatant was stored as described above and was assayed for crude protein content.

Blind test. A blind test was done for each measurement series. This differed from the sample in that it did not contain feed. Two parallels of the blind test were incubated in the same way as the feed samples. One of the sediments was used for digestion with pepsin and pancreatin.

Crude protein content was determined as described by the A. O. A. C. (1975). In the case of green lucerne the test materials were oxidized by Kjeldahl's method in the presence of selenium and copper sulphate catalyst, then, after vapour distillation, they were assayed for nitrogen content by titration with boric acid. For sulphuric acid destruction the whole amount of sample was used up. After the digestion of extracted sunflower meal, crude protein content was measured with a Kjell-foss instrument (oxidation: cc. H_2SO_4 + + H_2O_2, in the presence of Hg catalyst, distillation into NaOH solution, titration with 0.01 mol/l H_2SO_4 solution).

Calculation. "*Rumen undegradable protein*" (UDP) = crude protein content of the sediment of the first phase minus crude protein content of the blind test. "*Protein degraded in the rumen*" = crude protein content of 500 mg sample minus UDP. "*UDP digestible with pepsin*" = crude protein content of the second-phase supernatant minus crude protein content of the second phase of the blind test. "*UDP digestible with pancreatin*" = crude protein content of the third-phase supernatant minus crude protein content of the third phase of the blind test.

Determination of survival and proteolytic activity of rumen bacteria

Viability, multiplication, and maintenance of proteolytic activity of proteolytic bacteria contained by the rumen fluid used in the first phase were checked as follows.

Into each of 24 centrifuge tubes 500 mg extracted sunflower meal, 500 mg water-soluble starch and 40 ml McDougall's buffer were measured, then, after adding 10 ml filtered rumen contents CO_2 gas was bubbled through the mixture, and the tubes were closed. Twenty samples were placed in a water-bath of 38 °C and 4 samples were immediately centrifuged at 8000 *g*. The supernatant was discarded. Two sediments were immediately cultured for anaerobic bacteria and other two were stored at —20 °C until used for determination of protease activity.

Four of the 20 tubes were taken out from the water-bath in each of the 2nd, 4th, 6th, 24th and 48th h of incubation and processed as follows.

Anaerobic bacterial culture. One ml saline was measured onto each sediment, then from the upper layer bacteria were resuspended by gentle shaking, and from the suspension 10^{-6} and 10^{-7} dilutions were made. In a gloved chamber (Forma model 1029) 0.1 ml of both dilutions were spread on RGCA medium supplemented with 20% milk (Bryant and Burkey, 1953; Lányi, 1980). RGCA medium was prepared from 30 ml filtered rumen fluid, 0.0248 g glucose, 0.0248 g cellobiose, 0.05 g cysteine-HCl$\times H_2O$, 0.1 g $(NH_4)_2SO_4$, 0.4 ml rezazurine solution, 20 ml distilled water, and 50 ml salt solution.* Finally, agar was added to give a final concentration of 0.2%. The media were distributed into Petri dishes and sterilized.

Three parallel cultures were made from both dilutions of both incubation mixtures in a mixture of 5% CO_2, 10% H_2 and 85% N_2 at 39 °C for 48 h. Colonies with proteolytic activity, as indicated by clearing-up of the medium, were counted. Cultures in which the proteolytic zones had merged were regarded as non-interpretable since it could not be seen whether the individual colonies possessed protease activity.

Determination of protease activity

The sediments were resuspended in 10 ml 0.1 mol/l phosphate buffer (pH 6.5). The proteolytic activity of the mixture was characterized by its azocasein-degrading capacity (Baintner, 1981). The quantity of azocasein degraded by the proteases at 50 °C in 1 h was measured with a Spektromom 195 D photometer at 436 nm. Changes in protease activity during the incubation were determined using another 24 samples.

Determination of the degradability of green lucerne and extracted sunflower meal proteins

Both feeds were used in five parallel determinations (5×2 tubes) to demonstrate the functioning of the *in vitro* system.

To demonstrate reproducibility of the method, *in vitro* degradability of extracted sunflower meal was determined in all three phases using fresh rumen fluid obtained from the same animal 6 weeks later.

* Composition: 25 ml solution 1 (0.6 g K_2HPO_4 and distilled water to give 100 ml), 25 ml solution 2 (1.2 g NaCl, 1.2 g $(NH_4)_2SO_4$, 0.6 g KH_2PO_4, 0.12 g $CaCl_2$, 0.25 g $MgSO_4\times 7H_2O$, and distilled water to give 100 ml)

Results

Determination of survival and proteolytic activity of rumen bacteria

From the 10^{-6} dilution anaerobic bacteria were successfully cultured at all times, whereas from the 10^{-7} dilution at all times except two (Table I). In a large proportion of the cases there were so many bacterial colonies with proteolytic activity that proteolytic activity of the individual colonies could not be determined accurately because of fusion of the proteolytic zones (n.i., non-interpretable).

The 48-h culture of passaged colonies that had grown out under aerobic conditions gave negative results.

During incubation there was a significant increase in protease activity (Table II).

Determination of the degradability of feed proteins

The values measured during *in vitro* digestion of *green lucerne* are shown in Table III. On the average of 5 measurements, crude protein digestibility was 73.1% in the three phases. 86.5% (110.0 ± 7.9 g/kg dry matter), 7.1%

Table I

Anaerobic culture of proteolytic bacteria from phase-1 test materials incubated for different times

Time of incubation (h)	Parallels 1 dilution		2 dilution	
	10^{-6}	10^{-7}	10^{-6}	10^{-7}
0	n.i.	−	n.i.	+
	n.i.	−	n.i.	++
	++	+	n.i.	n.i.
2	n.i.	n.i.	+	−
	+	+	++	−
	n.i.	++	−	−
4	n.i.	+	n.i.	++
	n.i.	+	++	++
	n.i.	++	++	+
6	+	+	+	++
	n.i.	n.i.	+	++
	n.i.	+	+	−
24	n.i.	n.i.	n.i.	+
	++	n.i.	n.i.	−
	n.i.	−	++	−
48	n.i.	−	n.i.	n.i.
	n.i.	−	n.i.	n.i.
	++	−	n.i.	+

++ more than 15 colonies with proteolytic activity;
+less than 15 colonies with proteolytic activity;
n.i. = non-interpretable because of fusion of proteolytic zones

Table II

Protease activity values of rumen fluid incubated with extracted sunflower meal for different times

No. of parallel determinations	Experiment 1			Experiment 2					Mean of the two experiments
	1	2	Mean ± SEM	1	2	3	4	Mean ± SEM	
	Protease activity values (μg crystalline trypsin equivalent/ml)								
0	0.42	0.40	0.410 ±0.01	0.59	0.51	0.53	0.62	0.563 ±0.03	0.512 ±0.036
2	0.45	0.20	0.325 ±0.13	0.72	0.72	0.82	0.85	0.778 ±0.03	0.627 ±0.103
4	0.51	0.44	0.475 ±0.03	0.97	0.86	0.88	0.87	0.896 ±0.025	0.755 ±0.090
6	0.53	0.54	0.535 ±0.005	1.01	0.92	1.22	0.95	1.026 ±0.07	0.862 ±0.112
24	0.76	0.89	0.825 ±0.07	1.21	1.04	0.73	0.95	0.972 ±0.10	0.93 ±0.073
48	0.74	0.73	0.760 ±0.02	1.61	1.44	1.70	1.02	1.444 ±0.15	1.215 ±0.173

(9.1 ± 2.07 g/kg dry matter), and 6.4% (8.2 ± 2.8 g/kg dry matter) of the protein was degraded by the rumen fluid, pepsin and pancreatin, respectively.

In vitro digestibility of *extracted sunflower meal* (average of 5 measurements) was as follows (Table IV, first measurement): 68.3% (289.5 ± 10.2 g/kg dry matter), 17.7% (75.0 ± 0.0 g/kg dry matter), and 5.5% (23.5 ± 8.1 g/kg dry matter) was degraded by the rumen fluid, pepsin and pancreatin, respectively. Total crude protein digestibility averaged 91.5% during the three phases.

The digestion experiment was repeated 6 weeks later to determine reproducibility of the measurements. At that time crude protein digestibility of extracted sunflower meal (in the three phases, on average of 5 measurements) was as follows (Table IV, second measurement): 66.7% (282.8 ± 8.7 g/kg dry matter), 27.1% (115.0 ± 6.1 g/kg dry matter), and 5.1% (21.8 ± 4.3 g/kg dry matter). As a result of higher digestibility with pepsin, 98.9% of the crude protein was degraded during the three phases.

Table III

Digestibility of green lucerne protein as measured by the three-phase *in vitro* system

Crude protein content of feed g/kg	UDP		RDP		UDP digestible with pepsin		UDP digestible with pancreatin		Total digested crude protein	
	g/kg feed	crude protein %	g/kg feed	crude protein %	g/kg feed	crude protein %	g/kg feed	crude protein %	g/kg feed	crude protein %
174.0	50.0	28.7	124.0	71.3	11.5	6.6	4.8	2.8	140.3	80.6
174.0	40.0	23.0	134.0	77.0	11.5	6.6	0.8	0.5	146.3	84.1
174.0	77.0	44.3	97.0	55.7	10.7	6.1	11.0	6.3	118.7	68.2
174.0	77.0	44.3	97.0	55.7	10.8	6.2	7.0	4.0	114.8	66.0
174.0	76.0	43.7	98.0	56.3	0.8	0.5	17.4	10.0	116.2	66.8

UDP = rumen undegradable protein; RDP = rumen degradable protein

Table IV

Digestibility of extracted sunflower meal protein as measured by the three-phase *in vitro* system

Crude protein content of feed g/kg	UDP		RDP		UDP digestible with pepsin		UDP digestible with pancreatin		Total digested crude protein	
	g/kg feed	crude protein %	g/kg feed	crude protein %	g/kg feed	crude protein %	g/kg feed	crude protein %	g/kg feed	crude protein %
First determination										
424.0	118.2	27.9	305.8	72.1	75.0	17.7	8.4	2 0	389.2	91.8
424.0	164.0	39.4	260.0	61.3	75.0	17.7	50.4	11.9	385.4	90.9
424.0	118.0	27.8	306.0	72.2	75.0	17.7	33.6	7.9	414.6	97.8
424.0	154.4	36.4	269.6	63.6	75.0	17.7	16.8	4.0	361.4	85.2
424.0	118.0	27.8	306.0	72.2	75.0	17.7	8.4	2.0	389.4	91.8
Second determination										
424.0	139.4	32.9	284.6	67.1	125.0	29.5	16.8	4.0	426.4	100.6
424.0	146.8	34.6	277.2	65.4	125.0	29.5	8.4	2.0	410.6	96.8
424.0	127.2	30.0	296.8	70.0	100.0	23.6	25.2	5.9	422.0	99.5
424.0	149.6	35.3	274.4	64.7	125.0	29.5	25.2	5.9	724.6	100.1
424.0	143.0	33.7	281.0	66.3	100.0	23.6	33.6	7.9	414.6	97.8

UDP = rumen undegradable protein; RDP = rumen degradable protein

Discussion

Optimal protein supply of ruminants is inconceivable without testing methods suitable for monitoring degradation and digestion of feed proteins at different places of the digestive tract. In this respect, a method yielding data closest to those measured *in vivo* can be used best. The third phase of the procedure presented in this paper lessens the difference between the *in vitro*

and *in vivo* measured values of proteolysis. Even this method fails to give results numerically identical with those measured *in vivo*, since proteolysis in the ruminant digestive tract is influenced by a variety of factors including the animal species (Cippoloni et al., 1951; Fonnesbeck et al., 1981; Blankenship et al., 1982), feeding level (Alwash and Thomas, 1971; Robinson et al., 1985), preparation of the feed (Minson, 1963), and composition of the ration (Fonnesbeck et al., 1981; Minson, 1963). Some of these factors vary day by day; thus, only the mean degradability or digestibility of a given protein can be spoken of.

The most critical part of the laboratory method presented here is the first phase since in that phase rumen fluid containing live bacteria is used. During the collection and filtration of rumen fluid the bacteria which had until then lived under anaerobic conditions might come into contact with oxygen. Survival of the bacteria may also be endangered by a decrease of temperature. All these factors have prompted us to study the viability and proteolytic activity of the rumen microflora. The results have shown that rumen bacteria survive the preparatory processes without suffering any substantial damage and they maintain their growth capacity too. Large numbers of proteolytic bacteria grew out even from mixtures that had been incubated for 48 h.

It was a typical finding that protease activity increased parallel to incubation. During the first measurement this increase could be observed only for 24 h. At the second measurement proteolytic activity was found to increase throughout the incubation period. This increase may have been due to the marked bacterial growth that occurred in the first phase as a result of dilution of rumen fluid, adjustment of pH, and concentration drop of the end-products of bacterial metabolism. This growth lasted as long as sufficient amounts of nutrients (water-soluble starch, protein) were available in the incubation mixture.

Changes in proteolytic activity during incubation draw attention to the necessity of considering the actual incubation times if a comparative evaluation of results obtained by different *in vitro* studies is to be made.

Results of bacterial culture and protease activity determination indicate that the first phase of the *in vitro* method functions reliably: all 48 samples used in the two studies contained bacteria capable of proteolysis.

The three-phase method was used to predict the protein degradability of two different feeds. Table V shows a comparison of UDP values obtained by us with similar parameters published in the literature. For green lucerne UDP values between 20 and 49%, whereas for extracted sunflower meal those between 10 and 45% were reported. The values measured by us were always in the middle third of these ranges.

The two determinations of the protein content of extracted sunflower meal performed at two different times gave consistent UDP values (average

Table V

Comparison of UDP values (crude protein %) reported in the literature with those measured in the present study

Source	Feed	
	green lucerne	extracted sunflower meal
Jarrige (1978)	45	19—28
ARC (1980)	30—49	10—29
ARC Supplement (1984)	—	18—34—45*
Schingoethe (1984)	20—30	—
Preston (1985)	20	—
Madsen and Hvelplund (1985)	—	27
Veresegyházy et al. (1984)	—	32.6
Present study	36.8	31.7
	—	33.3

* depending on the feeding level

of 5 measurements: 134.5 $\pm$ 10.2 and 141.2 $\pm$ 3.9 g/kg dry matter, respectively). As related to the crude protein content of the feed this difference remained below 2% (31.7% and 33.3%). Reproducibility of the results was proved by the UDP value (32.6%) measured by us earlier (Veresegyházy et al., 1984).

The significance of the second and third phase in the estimation and evaluation of protein content was indicated by the fact that while the difference between green lucerne and extracted sunflower meal in UDP was only 4.3%, a much larger difference was found between the two feeds in the ratio of total crude protein degraded during the three phases (22.1%). Comparing the coefficients of variation of UDP values (green lucerne 12.3; extracted sunflower meal 7.6 and 2.8, respectively) with those of total protein degraded during the three phases (green lucerne 5.2, extracted sunflower meal 2.2 and 0.7, respectively), it appears that the latter values are always lower. Thus, introduction of the second and third phases rendered protein evaluation more accurate.

Values obtained within a given measurement series indicate that at least 4 or 5 parallel determinations are needed for the evaluation of a given feed. Fewer determinations pose the risk of obtaining less accurate results, while more determinations require unnecessary additional labour and costs, without appreciably improving the accuracy of the results.

The method uses rumen fluid incubated for 48 h instead of the so-called "0 min" blind control. The reason for this is that with the filtered rumen fluid proteins originating from sources other than the feed always get into the first phase. When degraded, these proteins serve as energy source for the bacteria and are utilized by bacteria living and multiplying in the sample. Inclusion in the UDP fraction of bacterial proteins of such origin, which are present in the centrifuged sediment, would constitute a significant source of error.

Accurate measurement requires that after the first-phase digestion the whole sediment be destroyed and assayed for nitrogen content, since the sediment is not homogeneous.

As compared to the original procedure, the advantages of the modified laboratory method recommended by us can be summarized as follows. (1) The method determines protein degradation by the rumen fluid instead of that of total organic matter. (2) The use of water-soluble starch renders the method suitable for evaluating high-protein and low-starch feeds as well. Owing to its high molecular mass and slower decomposition, starch probably provides energy longer and more uniformly than does maltose, a compound used by other authors (Broderick and Craig, 1980; Broderick, 1987). (3) Intact protein is available for digestion with pepsin (when using the method of Tilley and Terry (1963), drying at 105 °C after the first phase may affect protein digestibility). (4) By introducing the third phase, *in vitro* data may come closer to the actual values determined *in vivo*. (5) Analysis of the first-phase supernatants permits monitoring of the fate of crude protein dissolved by the rumen fluid (e.g. measurement of the quantity of NH_3, dissolved protein and peptide).

The method presented here is practical, suitable for serial determinations and for simulating abomasal and small-intestinal digestion in addition to protein degradation in the rumen.

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ACTIVITIES OF SOME SERUM ENZYMES IN HALOTHANE REACTED AND NON-REACTED PIGS

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Eight- and nine-week-old Hungarian Landrace pigs were tested with halothane as described by Laky et al. (1985). Immediately after the test blood samples were taken for determination of the activity of serum creatine kinase (CK), creatine kinase MB (CK-MB) isoenzyme, aldolase (ALD), lactate dehydrogenase (LDH) and α-hydroxybutyrate dehydrogenase (α-HBDH).

Elevated creatine kinase, creatine kinase MB isoenzyme and aldolase activities indicating enhanced susceptibility to stressors were found in 92% of the halothane reacted and 16% of the halothane non-reacted animals. In these individuals the activities of lactate dehydrogenase and α-hydroxybutyrate dehydrogenase were also high.

Data of the literature show a close relationship between enhanced susceptibility to stressors and halothane reaction in pigs. It was suggested, therefore, that determination of the activity of appropriate serum enzymes migh tbe used for detecting this enhanced susceptibility.

Keywords: Halothane susceptibility, serum creatine kinase, aldolase, pig.

Meat quality problems like PSE (pale, soft, exudative) or DFD (dark, firm, dry) are primarily due to the interaction of genotype and environment: the genetic trait will be manifested only if the animals are affected by adverse environmental factors above a definite threshold level (Kolb, 1979). Such factors are e.g. housing, transportation to the slaughterhouse, and the way of slaughtering (Addis et al., 1974; Lengerken and Pfeiffer, 1977; Moss and McMurray, 1979; Nielsen, 1979; Wittmann et al., 1981; Szilágyi and Takács, 1980; Szilágyi et al., 1981; Szilágyi et al., 1982; Jensen and Barton-Gade, 1985; Topel and Hallberg, 1985).

Several methods have been developed for detecting susceptibility to stressors: exposure to high ambient temperature, exercise or drugs was followed by determination of selected parameters (Nelson and Chausmer, 1981; Takala and Hassinen, 1981; Vihko and Salminen, 1983; Kauppinen et al., 1983; Kainulainen et al., 1984; Salminen et al., 1984; Fésüs et al., 1986).

By the so-called halothane test, piglets could efficiently be selected already at the age of 7 to 8 weeks (Schmitten and Schepers, 1979; Webb, 1980; Carden et al., 1985; Laky et al., 1985). The level of some serum enzymes, mostly of those originating from the striated muscle, was shown to be higher in the halothane reacted animals than in the halothane non-reacted ones.

Creatine kinase (CK) became more widely used than other enzymes, although its use for selection was impractical due to methodological problems (Augustini et al., 1979; Richter, 1979; Szilágyi and B. Kovács, 1979; Bickhardt and Richter, 1980; Schlenker et al., 1981; Schulman, 1980). However, in the literature we were unable to find data about the interrelationship between serum creatine kinase MB isoenzyme (CK-MB) levels and enhanced susceptibility to stressors.

In the present work we examined the relationship between enhanced susceptibility to halothane and the activity of some serum enzymes originating from, or functioning in, the striated muscle, in a population of 8- to 9-week-old Hungarian Landrace pigs.

Materials and methods

Eight- to nine-week-old Hungarian Landrace pigs were treated with halothane (Laky et al., 1985) for 1 to 4 min on two farms. Immediately after treatment blood was withdrawn from the jugular vein of 74 reacted animals (showing contraction of the skeletal muscles), from that of 64 animals that reacted atypically (breathing difficulties with or without slight spasm), and from that of 178 non-reacted control pigs. The activity of creatine kinase (CK), creatine kinase MB isoenzyme (CK-MB), aldolase (ALD), lactate dehydrogenase (LDH), and α-hydroxybutyrate dehydrogenase (α-HBDH) was determined in the sera using Boehringer test kits and an Eppendorf ACP 5040 equipment. Group means were compared by Student's *t* test.

Results

Enzyme activities of the experimental groups are shown in Table I. In the *halothane reacted* group all the activities were higher than in the control group; the differences reached the level of significance ($P < 0.001$) for CK, CK-MB and ALD. When these values were considered *individually*, in 92% of the halothane reacted pigs (in 68 out of 74 animals) pathologically high (>600 U/l) serum enzyme activities were found. From pigs with an unusually strong reaction to halothane, blood was repeatedly taken; in these cases serum enzyme activities tended to increase.

In 48 % of the pigs that had given an atypical reaction to halothane, the CK (715 ± 423 U/l) and ALD (27 ± 32 U/l) activities surpassed those of the control animals.

In the halothane *non-reacted* group ($n = 178$) 29 pigs (16%) were found whose enzyme activities approximated those of the halothane reacted animals (CK: 1555 ± 1256; ALD: 37.4 ± 23.7; LDH: 894 ± 342; α-HBDH: $552 \pm \pm 230$ U/l).

Table I

Serum enzyme activities (mean ± SD) in halothane reacted (Hal +) and non-reacted (Hal −) pigs

	CK	CK-MB	ALD	LDH	α-HBDH
			(U/l)		
Hal + (n = 74)	1515* ±828	179* ±92	42.1* ±77.6	788 ±319	504 ±217
Hal − (n = 178)	428 ±233	55 ±20	16.9 ±14.5	634 ±241	399 ±165

* Significant differences between groups: $P < 0.001$

Discussion

Stress may result, among other effects, in an increase of cell permeability and consequent release of intracellular enzymes into the circulation (Kolb, 1979).

In the present work treatment with halothane was used as stressor. Two of the enzymes determined (ALD and LDH) function in glycolysis, the determination of α-HBDH in relation to total LDH has great value in the measurement of the "heart" fractions of LDH isoenzymes, while CK provides energy for muscular contractions.

Creatine kinase consists of two monomers in three possible combinations (BB, MB, MM). Usually the BB isoenzyme occurs in the nervous tissue, and the MM isoenzyme is found in the adult skeletal muscle, although MB and sometimes BB isoenzymes have also been detected in the skeletal muscle of several species. The adult heart muscle is characterized by MM and MB isoenzymes (Cepica and Jorgensen, 1977).

In the present work big (significant) inter-group differences were found in the activity of CK, CK-MB and ALD. CK and ALD are enzymes originating primarily from the skeletal muscle, and indicate increased permeability of the muscle cells in halothane reacted animals. The ration of CK-MB isoenzyme to total CK was nearly the same in both groups, which is inconsistent with the occurrence of major lesions in the heart muscle.

In halothane sensitive pigs similarly high CK activities (1987 ± 1273 U/l) were found by Schulman (1980), while the CK activity of boars at the age of 100 days was 213 ± 88 U/l (Schlenker et al., 1981).

The positive, 92% correlation between halothane reaction and elevated enzyme activity, and the very activities found in repeatedly taken serum samples suggest that the determination of CK and ALD may be used for testing susceptibility to stressors.

Pathologically high enzyme activities were found in 29 out of the 178 halothane non-reacted pigs (16%). Although injuries can elevate the serum levels of enzymes of muscular origin, the present cases can be considered marginal halothane susceptibility without manifestation in clinical symptoms. In other words, they might represent individuals susceptible to stressors but not detected by the halothane test.

It is suggested that the determination of serum enzymes of muscular origin may be an aid in culling pigs susceptible to stressors.

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SHOT BIOPSY IN VIEW OF MUSCULAR DISORDERS IN PIGS

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Although a number of problems in connection with the use of shot biopsy have yet to be solved, it can be stated that by this technique biopsy samples of sufficient quantity (up to 1.5 g) can be obtained from the musculus longissimus dorsi of pigs of about 80 kg liveweight. The technique offers data on the metabolic status (biophysical and biochemical values) of muscles, provided that the postmortal processes have been simulated (incubation of bioptate).

The use of sensitivity tests on isolated subcellular fractions of the bioptate can lead to more precise results and consequently, to a remarkable reduction in the incidence of PSE syndrome (pale, soft, exudative meat).

The first results achieved under conditions of Slovakia (Czechoslovakia) in testing boars show that this method could find a permanent use in the ptactice.

Research institutions in a number of countries are now investigating possibilities of using shot biopsy for prediction of skeletal muscle disorders in pigs and it is excepted that many new results will be published soon.

Keywords: Biopsy, metabolism, muscular disorders, pig.

It is a well-known fact that the reaction of pigs to environmental stress is a complex phenomenon influencing a large number of economically important traits. Examples of these are reduction in meat quality, losses during fattening or transport, and impaired fertility.

Pigs susceptible to Porcine Stress Syndrome (PSS) or to malignant hyperthermia (MH) show a high incidence of pale, soft and exudative (PSE) muscle after slaughter.

It was also demonstrated that the syndrome could be triggered in these animals not only by stress but also by breathing the anaesthetic halothane (Eikelenboom and Minkema, 1974). Researchers have focused their efforts on methods to predict "stress susceptibility" and the potential for abnormal meat quality in live animals. Of these methods, halothane test is the most frequently used procedure in a large variety of systems. It is often combined with blood typing to identify halothane negative (HN) individuals and, in some instances, blood typing even replaces the halothane test. CK test (determination of creatine kinase activity) is less frequently used. Other methods, such as muscle biopsy, are still at an experimental stage, although tests for MH, the halothane or caffeine contracture tests on isolated muscle strips sampled by biopsy, have proved to be more accurate and useful in human medicine.

The diagnosis of MH in pigs by *in vitro* contracture tests appears to be much more difficult than in man, and the situation is further complicated by the occurrence of the PSE muscle condition in both MH-susceptible and MH-resistant pigs (Mitchell and Heffron, 1982). Since the primary objective of the breeder is to eliminate all potential PSE pigs from the herd, this limitation of the halothane test needs to be more widely appreciated. From this point of view, *in vitro* contracture tests would probably have the same limitation, in addition to their high costs and surgical risks.

This short review deals mainly with recent works published on the premortal metabolism of skeletal muscle in pigs in relation to MHS and the incidence of lower meat quality (PSE syndrome).

Biopsy and prediction of meat quality in pigs

According to Sybesma and Eikelenboom (1978), biopsy methods such as the use of biopsy needles and the results of muscle analysis (correlation coefficients of the substrate contents and parameters of meat quality) do not provide possibilities sufficient for practical use.

The results of shot biopsy, as described by Schöberlein (1976), proved that biopsy of a skeletal muscle would show better prospects of practical use. Shot biopsy is carried out by use of a common slaughter pistol specially adapted for this purpose: namely it has a cannula on the top designed for sampling fat and muscle tissues. Samples are taken by a "shot-out", and neither fixation nor anaesthesia (total or local) is needed. Lahučký et al. (1980*a*, *b*) further improved the shot biopsy technique so that no injury to the spine can occur, and samples can be taken even from pigs of lower liveweight (50 kg). The latest design of the cannula for sampling muscle and fat from the musculus longissimus dorsi (m. l. d.) provides for taking up to 1.5 g of muscle tissue from an 80-kg animal. A number of works on the use of shot biopsy and the results have been published by workers from Leipzig, German Democratic Republic (Hennebach, 1977; Hennebach et al., 1980; Hennebach and Lengerken, 1980).

When postmortal processes within the muscle bioptate were simulated by a 45-min incubation at 37 or 39 °C and several parameters were investigated afterwards, some significant relationships were found among the pH value, CPK activity of the juice pressed out of the bioptate, and postslaughter quality parameters. Evaluation of CPK activity in the juice pressed out of the bioptate, in spite of its clear relation to meat quality, seems to be rather time consuming and complicated. Hennebach and Lengerken (1982) also pointed out that this juice did not always show measurable CPK values.

As shown in our previous works concerning muscle metabolism in pigs (Lahučký et al., 1980*b*; Kováč et al., 1981), an incubation for 1 h at 39 °C in a

humid environment with subsequent determination of the pH value and/or R value of the bioptate and of the relationship of absorbance at 250 nm (inosine nucleotide) to absorbance at 260 nm (adenosine nucleotide; Honikel and Fischer, 1977) proved to be useful for evaluation of meat quality. It was also shown in other works (Lahučký et al., 1982) that statistically highly significant relationships can be found between pH_1 value of the meat (m. l. d.) and various biochemical parameters. Table I shows that in those experiments (carried out mainly in German Landrace pigs) the R value was very useful for the prediction.

Table I

Correlation coefficients between selected biopsy values and pH in the m. long. dorsi and m. semimembranosus 45 min post mortem (Lahučký et al., 1982)

Values		n	m. long. dorsi	m. semimembranosus
pH_1 value (bioptate)		70	0.65^{+++}	0.49^{+++}
R value		51	-0.73^{+++}	-0.67^{+++}
Meat liquid surface cm^2		61	-0.47^{+++}	-0.42^{+++}
ATP	µmol/g	51	0.58^{+++}	0.63^{+++}
G-6-P	µmol/g	49	-0.57^{+++}	-0.30^{+}
Glucose	µmol/g	49	-0.60^{+++}	-0.32^{+}
Lactate	µmol/g	51	-0.62^{+++}	-0.62^{+++}
Glycogen	µmol/g	51	0.56^{+++}	0.60^{+++}

$^{+}$ $P \leq 0.05$ $^{+++}$ $P \leq 0.001$

The existing results show that the level of correlations among selected biophysical and biochemical factors of the bioptate and meat quality (pH_1) is influenced also by genetic factors (breed, cross-breeds). Hennebach and Lengerken (1982), Pfeiffer and Lengerken (1984) and other authors reported an influence of the neurohormonal status of the pig during biopsy and at slaughter. Considering an equal load at the time of biopsy and at slaughter and using the meat quality calculation indices of Mäder (1974), Hennebach and Lengerken (1982) found a close correlation between the biopsy index and the meat quality index ($r = 0.88$). The status of the blood system e.g. hyperaemia at the biopsy site after previous shaving of the hairs (Lahučký, unpublished observation) can significantly influence the result of the biopsy. Non-standard working conditions (temperature, methods of approach) have a similar effect.

The results published by Fischer and Augustini (1984) also show that differences among selected substrates in bioptates with different meat quality are statistically highly significant. Correlations between parameters of the bioptate (substrates) and pH_1 of the m. semimembranosus (Table II) are similar to those reported earlier (Lahučký et al., 1982) in pigs of 80 kg liveweight (Table I). Relatively lower correlations of the m. l. d. with pH_1 have

yet to be clarified because they lead to a controversy concerning heterogeneity of the muscle tissues *in vivo*, although no differences were found between the right and the left side of the m. l. d., either without (Sybesma and van der Wal, 1974) or with an incubation of the bioptate (Lahučký et al., 1982).

Table II

Correlation coefficients between selected biochemical values of bioptate at various liveweights and pH_1 value in the m. longissimus dorsi and m. semimembranosus (n = 116) in pigs of different liveweight (Fischer and Augustini, 1984)

Values	Live-weight (kg)	pH_1 m. l. dorsi	pH_1 m. semimembranosus
Glycogen	50	0.33	0.26
	70	0.39	0.52
	90	0.50	0.47
Lactate	50	−0.35	−0.37
	70	−0.44	−0.57
	90	−0.36	−0.44
ATP	50	0.42	0.47
	70	0.47	0.62
	90	0.49	0.55
R value	50	−0.39	−0.41
	70	−0.48	−0.63
	90	−0.46	−0.56

Table III

Values of glycolytic metabolism in bioptate after 60-min incubation in H(+) and H(−) pigs (Fischer, 1985)

Value		H(+) (n = 76)	H(—) (n = 40)	*t* test
Glycogen, μmol/g	$\bar{x}$	26.1	38.2	+++
	s	10.1	11.8	
Lactate, μmol/g	$\bar{x}$	100.0	81.2	+++
	s	14.7	20.7	
ATP, μmol/g	$\bar{x}$	0.54	1.57	+++
	s	0.52	1.00	
R value	$\bar{x}$	1.29	1.11	+++
	s	0.10	0.15	

+++ $P \leq 0.001$

Metabolism of skeletal muscle in relation to the halothane and creatine kinase tests

Simulation of the postmortal processes in the muscle bioptate (temperature, time) enabled differentiation between halothane-positive and halothane-negative pigs of the German Landrace breed on the basis of biochemical parameters (Table III; Fischer, 1985). The tendencies (Lahučký et al., 1982) leading

towards a decreasing meat quality in halothane-positive pigs and towards a higher meat quality in halothane-negative ones require further verification, although Cheah et al. (1986) observed an increase of m. l. d. mitochondrial phospholipase A_2 activity in pigs of a halothane-sensitive line starting from about 12 weeks of age. However, the activity of this enzyme in a halothane-sensitive line remained almost constant during the growth period.

Lengerken et al. (1986) differentiated halothane-negative ($n = 126$) and halothane-positive ($n = 86$) pigs by submitting pH_0 and pH_1 values of the bioptate (incubation for 45 min at 37 °C) to the calculation of the relative load (u value). Thus, they took into account the influence of different conditions of the load on individual days of the experiment. The authors, however, did not succeed in differentiating halothane-negative pigs (homozygotes, heterozygotes) when using the pH_0 and pH_1 of the bioptate. In a recent work, Lengerken and Pfeiffer (1988) report highly significant differences in the bioptate values pH_{30} and R_{30} (incubation of the bioptate for 30 min at 39 °C) between halothane-positive and halothane-negative animals. The authors also mention the suitability of the biopsy method for differentiation of halothane-sensitive animals at very low and very high halothane frequencies.

On the other hand, without simulating the postmortal processes in the bioptate (i.e. without its incubation) the differences in selected biochemical parameters (substrates) between H^+ and H^- animals may not be apparent (Hennebach and Lengerken, 1980). Similarly, Honikel and Kim (1985) failed to find any difference in the ATPase activity of the myofibrillar fraction of bioptate (without incubation) between H^+ and H^- animals, although they reported some relation between ATPase activity (bioptate) and water-binding capacity of the meat.

From the results of comparing the halothane test, the CK test and shot biopsy as reported by Kováč et al. (1985) it appears that the results gained after biopsy, incubation of the bioptate (39 °C, 1 h) and pH measurement by use of a contact electrode are more appropriate for the prediction of meat quality than is determination of CPK activity. It also appears from previous results (Lahučký et al., 1982) that even by use of a standardized load (Myostress), correlations such as reported by Bickhardt and Richter (1980) can only be achieved when the CK activity of the blood is related to pH_1 of m. semimembranosus ($r = 0.49$). The correlations of CK and pH_1 of the m. l. d. were lower ($r = 0.31$).

To elucidate the aetiology of malignant hyperthermia, biochemical and physiological studies have been conducted both in human patients and in pigs. The results, particularly those of the pharmacological tests with halothane, caffeine and ionophore A 23187 (Reiss et al., 1986), indicate that the direct cause of this acute syndrome is an abnormal sustained increase of Ca^{2+} in the skeletal muscle fibre cytoplasm.

It is yet to be definitely established which one (or more) of the organelles of the MH muscle fibre is/are responsible for elevated intracellular calcium (Lopez et al., 1985). Cheah et al. (1986) have reported an anoxia-induced increased calcium release from mitochondria of the porcine MH muscle. According to Cheah, phospholipase A_2 is involved in the development of porcine malignant hyperthermia and it may be capable of influencing the sarcoplasmic reticulum to release Ca^{2+}. This assumption has recently been supported by findings on human malignant hyperthermia (Cheah, 1987). Other authors (O'Brien, 1986; etc.) reported that malignant hyperthermia might be induced by a hypersensitive calcium release mechanism and may be diagnosed by calcium, magnesium, ATP and caffeine sensitivity tests on isolated sarcoplasmic reticulum.

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DIRECT RADIOIMMUNOASSAY OF SERUM PROGESTERONE FOR MONITORING THE REPRODUCTIVE STATUS OF LARGE PIG HERDS

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The reproductive status of sows was monitored by serum progesterone radioimmunoassay (RIA) in a closed pig herd of 1100 sows, producing for the market. The method proved suitable for monitoring reproductive events (progesterone production during lactation, effect of weaning on the resumption of cycling, successful and unsuccessful inseminations, abortion, etc.) and for diagnosing ovarian disturbances (acyclia, irregular cycle, etc.). A total of 813 inseminations were followed up to determine the time of return to oestrus. The serum progesterone values of blood samples taken from the v. cava cranialis on day 22 after insemination indicated that pregnancy was diagnosed with a reliability of 75.5%, while non-pregnant sows failing to return to oestrus were identified with a reliability of 96.1%. The economic benefits to be derived from the use of this method in a pig herd are discussed.

Keywords: Serum progesterone, direct RIA, reproductive status, monitoring, sow, pig herd.

In previous communications (Faredin et al., 1987; Antal et al., 1987*a*) we reported on the determination of progesterone in bovine serum and milk, a method suitable for monitoring the normal and pathological oestrous cycle in cows, as well as for detecting inseminated but non-conceived and non-return cows (Antal et al., 1987*b*).

Experience gained during the above studies prompted us to investigate whether the serum progesterone levels can be used as indicators of pregnancy, abortion, acyclia or irregular cycles in sows. Subsequently, the suitability of this procedure for early pregnancy diagnosis and for demonstrating "empty" status in sows was examined.

Materials and methods

The studies were performed in a closed, specialized pig operation of 1100 sows of the Agricultural Combinate of Mezőhegyes.

From the vena cava cranialis of sows selected for the experiment blood samples were taken once or twice weekly.

In the period of study the times of oestrus and insemination were recorded regularly. From sows failing to return to oestrus by day 22 after insemination a blood sample was taken on day 22.

Sera separated by centrifugation were stored frozen at —15 °C until processed. Serum progesterone concentration was determined by the direct radioimmunoassay (RIA) procedure described earlier (Faredin et al., 1987).

Results

First the serum progesterone level of two healthy sows (Gomb, no. 409 and Csipke, no. 490) was monitored over three cycles and after successful insemination. The serum progesterone levels of these sows showed regular fluctuations according to a 21-day cycle. After successful insemination they remained at a high level, indicating pregnancy.

The serum progesterone concentration of sow no. 6636 (Fig. 2) indicated the absence of ovarian cyclicity during suckling. Ovarian function was resumed only on days 4–5 after weaning. Subsequently, after insemination the sow conceived.

Figure 3 shows the serum progesterone level of a sow successfully inseminated at the first oestrus after weaning (no. 4745) throughout pregnancy.

Serum progesterone levels measured after an unsuccessful and a subsequent successful insemination are presented in Fig. 4 (sow no. 4719). The unsuccessful insemination was followed by a regular cycle. Subsequently, after the successful insemination the serum progesterone level kept rising continuously throughout gestation.

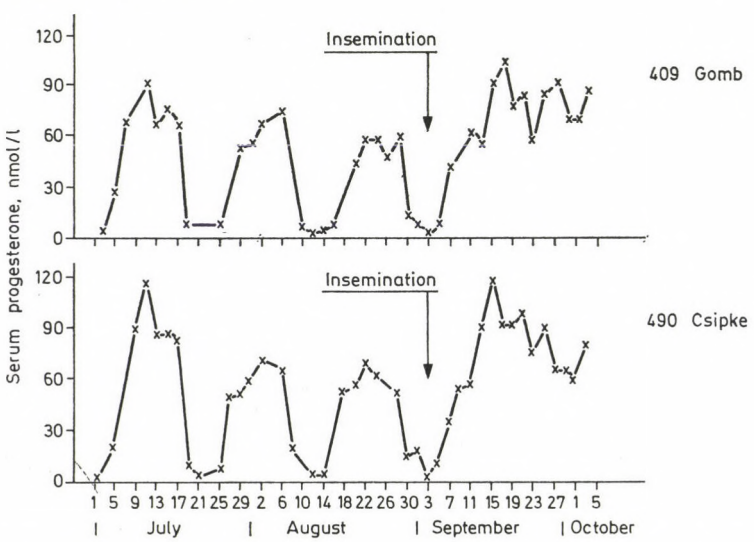

Fig. 1. Serum progesterone level in sows during three regular cycles and after successful insemination

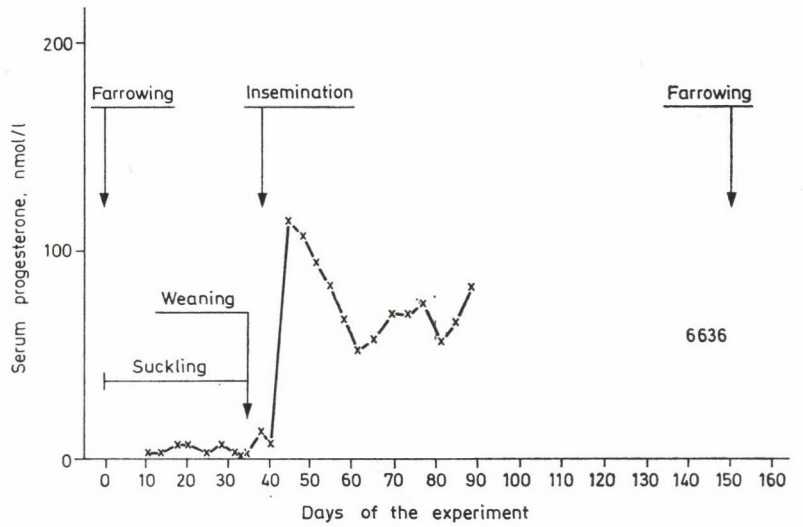

Fig. 2. Serum progesterone concentration in a sow during suckling and after successful insemination following weaning

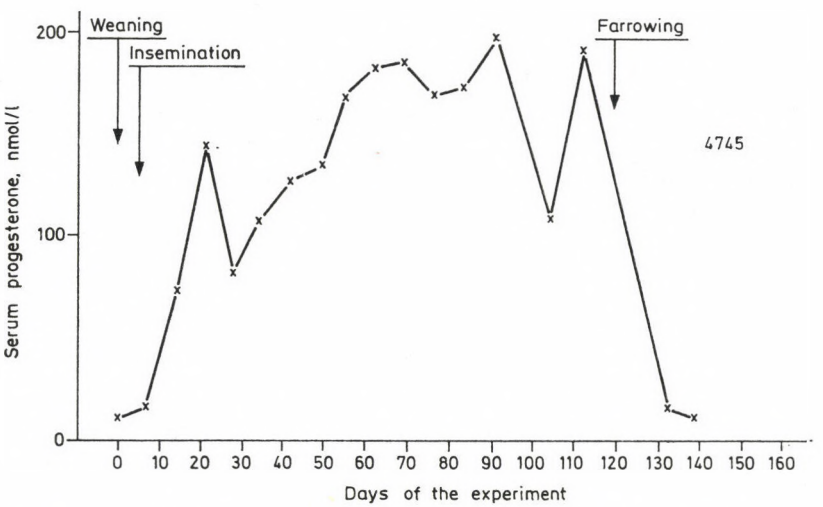

Fig. 3. Serum progesterone concentration of a sow throughout pregnancy

Figure 5 shows the serum progesterone levels of a sow (no. 230) that conceived after successful insemination but aborted subsequently. The rising serum progesterone level reflects pregnancy. After abortion the serum progesterone level abruptly fell down.

The serum progesterone levels of a sow (no. 231) that failed to come into oestrus for 36 days after weaning are shown in Fig. 6. During that period

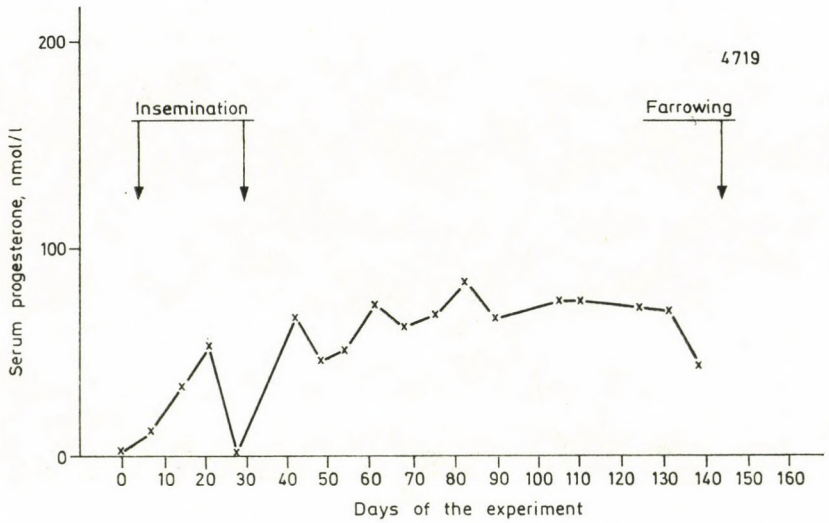

Fig. 4. Serum progesterone concentration in a sow after successful insemination

serum progesterone level remained below the detectability limit. Acyclia was followed by irregular ovarian cycles, as shown by the progesterone assay.

Figure 7 demonstrates the serum progesterone levels of a sow (no. 4701) with irregular cycles. The length of the cycles observed was 4, 3, 6 and 5.5 weeks, respectively. Regular cycles were observed only at the time of insemination: subsequently, no oestrus was demonstrable by the methods currently used under large-scale conditions.

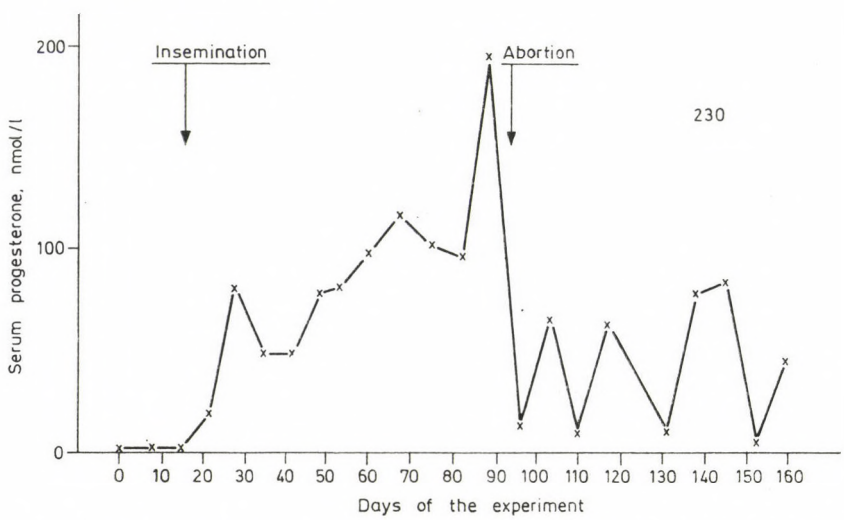

Fig. 5. Serum progesterone concentration of a sow after successful insemination and after abortion

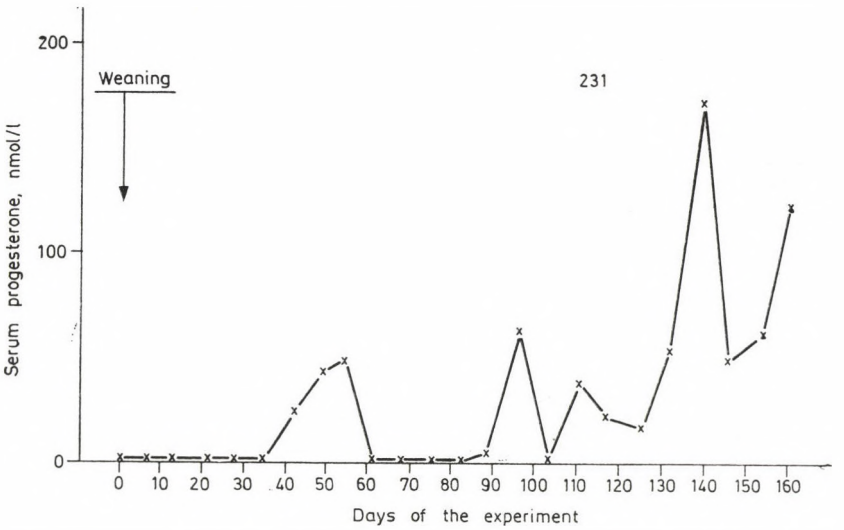

Fig. 6. Serum progesterone concentration of a sow with oestrous cycle disorder (acyclia)

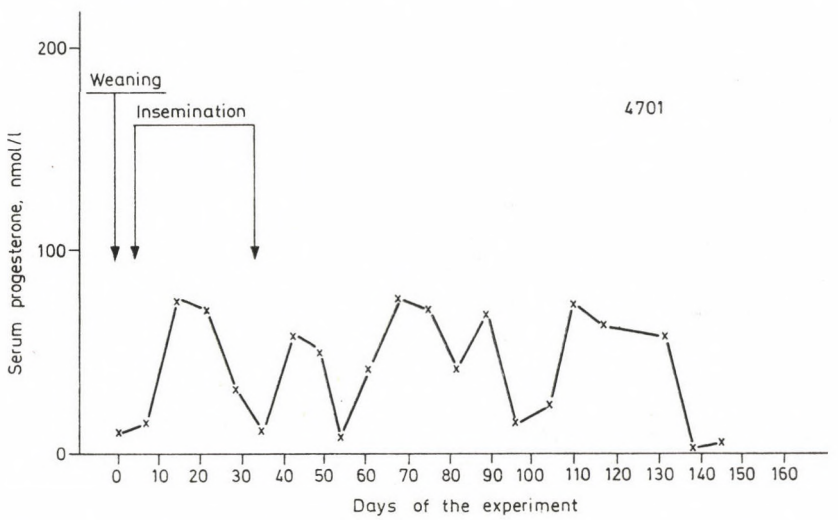

Fig. 7. Serum progesterone concentration of a sow with oestrous cycle disorders

With knowledge of these results an attempt was made at using serum progesterone analyses for detecting inseminated but non-conceived and non-return sows. A total of 813 inseminations were performed on the farm in the period of study (in September and October). Fifty-six out of the 813 inseminated sows (6.9%) returned to oestrus. From these sows no blood samples were taken for early pregnancy diagnosis.

The remaining 757 sows did not return to oestrus by day 22 after insemination. From these sows blood samples were collected and assayed for progesterone (Table I).

Sows with serum progesterone concentrations exceeding 31.8 nmol/l (10 ng/ml) were classified as *pregnant*. A total of 603 sows belonged to this group (79.7%): of them, 455 animals (75.5%) actually farrowed.

One hundred and forty-eight sows (24.5%) considered pregnant on the basis of the serum progesterone level measured on day 22 after insemination failed to farrow. This was due to irregular oestrus cycles, cystic ovarian degeneration (a disorder fairly common under conditions of closed, large-scale management technology), lutein cysts, delayed regression of the corpus luteum and ovarian atrophy, or to early fetal death, which was indicated by postponed return to oestrus. Twenty-four sows of this group were culled or died, and two sows aborted their fetuses.

Sows with serum progesterone values below 31.8 nmol/l (10 ng/ml) were considered non-pregnant, i.e. "empty" (Table I). A total of 154 sows (20.3% of the sows tested) fell into this category. Of them, 6 sows (3.9%) farrowed within 120 days as of the time of insemination and 148 animals (96.1%) proved actually non-pregnant.

These data show that by serum progesterone assay on day 22 after insemination the unsuccessfully inseminated, i.e. non-pregnant, sows can be detected most reliably.

To illustrate the magnitude of economic benefit to be derived from progesterone assays, Table II presents the subsequent reproductive events of the 154 sows that were identified as non-conceived among the 813 inseminated sows. Fifty-four sows returned to oestrus already in the first cycle following insemination (on day 24.3 on the average). In the case of these animals no

Table I

Correlation of serum progesteron concentration and reproductive status in sows

Grouping by serum progesterone level	No. of sows tested	Sows failing to farrow						
		Sows returning to oestrus in cycle			Sows not returning to oestrus	Culled or died	Aborted	Farrowed
		1	2	3				
Above 31.8 nmol/l (10 ng/ml) PREGNANT	603 79.7%	28	18	14	62	24	2	455 75.5 %
Below 31.8 nmol/l (10 ng/ml) NON-PREGNANT	154 20.3%	54	26	3	52	13	—	6 3.9 %
Total:	757	82	44	17	114	37	2	461

Table II

Calculation of economic benefit to be derived in the sow herd tested on the basis of results obtained for sows diagnosed as non-pregnant by serum progesterone assay on day 22 after insemination

Number of sows returning to oestrus		No. of days elapsing until return to oestrus (mean)	No. of days that can be saved by progesterone assays
In the first cycle:	54 sows	24.3	$(42.4 - 22) \times 26 = 530.4$
In the second cycle:	26 sows	42.4	$(63.7 - 22) \times 3 = 125.1$
In the third cycle:	3 sows	63.7	
No. of sows not returning to oestrus up to day 115 after insemination:	52 sows	—	$(115 - 22) \times 52 = 4836.0$
Culled:	13 sows	—	—
Farrowed:	6 sows	—	—
Total number of feeding and management days saved:			5491.5

appreciable benefit was derived from the serum progesterone assay. Twenty-six sows returned to oestrus only in the second cycle (on day 42.4 in average): in these animals the serum progesterone assay saved 20.4 days per sow, on the whole 530.4 days. Three sows came into oestrus only in the third cycle (on day 63.7 in average): in their case 125.1 days were saved by the serum progesterone assay.

By the serum progesterone assay the most significant results were achieved in the 52 sows which failed to return to oestrus for 115 days after an unsuccessful insemination. In this group the serum progesterone assay indicated the sows' non-pregnant status 93 days earlier (total savings for the group: 4836 days). In the period of study 13 sows were culled and 6 sows farrowed. The latter 19 sows were excluded from the evaluation of results. As shown in Table II, in the case of the 813 inseminated sows the serum progesterone assay shortened the reproduction time by 5491.5 days. This corresponds to 6.76 days per sow. Considering the number of sows used in the study and a sow rotation of 2.2, the annual savings would amount to 15.4 days per sow.

Discussion

Use of serum progesterone analysis for sows was first reported by Robertson and Sarda (1971). In the first phase of the present study the applicability of direct serum progesterone RIA (Faredin et al., 1987) for monitoring reproductive events in sows was studied. In sows with a regular oestrous cycle

the serum progesterone levels keep rising from oestrus up to day 16–17 of the cycle, when they abruptly decline. This is consistent with the findings of Parvizi et al. (1976).

Serum progesterone concentration persists at a high level following a successful insemination and drops markedly only immediately before farrowing. During lactation the serum progesterone concentration is very low. By studying the serum progesterone concentration of Large White sows at farrowing and during lactation, Ash and Heap (1975) obtained similar results.

In the present study, successful insemination was followed by permanently high serum progesterone level. As a result of abortion, however, an abrupt, steep decline of the serum progesterone level was observed.

Irregular oestrous cycle was diagnosed in a sow with permanently low, then irregularly fluctuating, serum progesterone level. In another sow the cycle was somewhat lengthened (4 weeks) and unsuccessful insemination was followed by irregular cycles lasting 4, 3, 6 and 5.5 weeks, respectively. These cycles were easy to monitor by the serum progesterone assay.

The applicability of serum progesterone assays for early pregnancy diagnosis in sows was studied by numerous researchers (Ellendorf et al., 1976; Hultsch et al., 1979; Lampe et al., 1982; Schneider, 1978; Schneider et al., 1982; Schneider et al., 1984; Tillson et al., 1970) including Hungarian authors (Antal et al., 1983; Mézes et al., 1986).

With regard to the fact that ultrasound pregnancy testing can be used efficiently only beyond day 30–42 after insemination, attempts are constantly being made at developing methods suitable for detecting the pregnant or non-pregnant status of sows within the shortest possible time after insemination. Cunningham et al. (1983) achieved promising results in sows by serum oestrone sulphate determination between day 25 and 29 after insemination. By enzyme-immunoassay (EIA) of serum oestrone sulphate Sugiyama et al. (1985) diagnosed pregnancy with 100% accuracy and detected non-pregnant sows with a reliability of 78.6%. By a single plasma progesterone determination between day 16 and 24 after insemination, Ellendorf et al. (1976) diagnosed pregnancy with a reliability of 96.4%. The difficulty of blood sampling was mentioned as a drawback of plasma progesterone assay. Hultsch (1980) analysed 8947 samples by plasma progesterone assay and found the reliability of pregnancy diagnosis 91.1%, while that of detecting non-pregnant sows 96.7%. Lampe et al. (1982) tested 4589 gilts for pregnancy by the plasma progesterone competitive protein binding assay. The reliability of pregnancy diagnosis was 79.6% and that of detecting non-pregnant animals 96.8%. According to Schneider et al. (1984) the best time for the reliable diagnosis of non-pregnant status of sows by progesterone analysis is on day 22 or 23 after insemination.

Hungarian authors (Mézes et al., 1986) determined the plasma progesterone concentration of a small number of sows by RIA following extraction and

found the error of pregnancy diagnosis 9.68%. The error of diagnosing the non-pregnant status was unacceptably high (17.65%).

In this study, 56 out of the 813 inseminated sows returned to oestrus by day 22 after insemination. From these sows, therefore, no blood samples were taken for progesterone assay.

Our procedure showed that 154 out of the 757 sows (20.3%) failing to return to oestrus by day 22 after insemination proved non-pregnant and only 6 out of them (3.9%) farrowed in the period of study lasting 115 days as of the time of insemination. Within the sow group diagnosed "non-pregnant" there were rather many sows (n =52) that failed to return to oestrus throughout the period of study. By the serum progesterone assay primarily such animals can be identified at the earliest possible time after insemination.

In summary: by serum progesterone assay on day 22 after insemination non-pregnant sows can be detected with a reliability of 96.1%. For the 813 inseminations followed up in this study, the introduction of serum progesterone assay in practice would have shortened the reproduction time by 5491.5 days (6.7 days per sow). This would render possible substantial savings in terms of feeding and management costs.

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SIMULTANEOUS FLUOROMETRIC DETERMINATION OF VITAMINS A AND E IN SERUM

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A simple fluorometric method suitable for serial determinations is reported for simultaneous determination of vitamins A and E in serum.

The solvents are pretreated chemically and by distillation. After shaking with ascorbic acid in ethanol, the vitamins are extracted from the serum samples with petroleum ether. The extract is used for the determination.

The sensitivity of the method is 20 μg/100 ml for vitamin A and 0.04 μg/ml for vitamin E. Recovery rates are between 87 and 106%.

The method was tested on a total of 160 serum samples of healthy animals belonging to 8 different species. The results showed good agreement with the so-called reference values published in the literature.

Keywords: Simultaneous determination, fluorometry, vitamin A, vitamin E, serum.

The importance of determining the supply status of animals with the essential vitamins A and E has long been known. For decades, colorimetric and spectrophotometric methods were used (Knobloch, 1963): these procedures are, however, laborious, time consuming, and require large quantities of chemicals. A major advancement was made possible by up-to-date instrumental analytic procedures such as fluorometry and liquid chromatography. Our choice fell on fluorometry which is simple, rapid, suitable for serial determinations, and more sensitive than any of the procedures used earlier.

The analytical literature contains some papers on fluorometric vitamin determination in human serum samples (Hansen and Warwick, 1978; Thompson et al., 1973; Driskell et al., 1985). The method presented here is a combination of these procedures. In our procedure the optimum solvent ratio, solvent quality, duration of operation, and wavelengths of fluorescence determination etc. are determined at every step of the procedure, in a series of parallel measurements, by adding standard vitamin solutions.

Materials and methods

1. *Pretreatment of solvents, washing-up of glass tubes*

(a) Distilled water with sodium ascorbate: Titrate 1% ascorbic acid with 1 N NaOH until its pH reaches 4.5. Use twice distilled water for solution making. Collect the solution in a glass bottle through a glass funnel. The solution is stable for one month.

(b) Ascorbic acid in ethanol: Reflux absolute ethanol with 1 g $KMnO_4$ + + 2 g KOH per litre for 30 min, then distil it. When stored in a brown bottle, the solution ("pretreated ethanol") is stable for one month.

Add 10% aqueous ascorbic acid solution to the pretreated ethanol to give a final concentration of 0.1% ascorbic acid.

(c) Petroleum ether: Distil petroleum ether (boiling point: 40 to 70 °C). Discard 100 ml pre-distillate and 100 ml post-distillate per litre and use only the main fraction of the distillate. Store the solution in a brown bottle.

(d) Washing-up glass tubes and brown bottles: Soak new glass vessels in chromsulfuric acid and used ones in Calgon inorganic detergent or RBS 25 solution (Fluka) overnight. Rinse the bottles thoroughly with tap-water 10 to 12 times, with distilled water 6 to 7 times, with twice distilled water 4 to 5 times, and with pretreated ethanol twice, then dry them.

2. *Preparation of standard solutions*

(a) *Standard vitamin A solution.* A stock solution of 1 mg/ml concentration is prepared, using measurement according to weight, in pretreated ethanol. 1 IU = 0.344 μg vitamin A acetate. E.g. one g of the oily vitamin A preparation (Merck) contains one million IU = 344 mg vitamin A acetate. 50 mg pure vitamin A acetate equals 0.1453 g oily vitamin A acetate solution. Dissolving this in 50 ml pretreated ethanol, a stock solution of 1 mg/ml concentration is obtained.

By diluting the stock solution with pretreated ethanol in several steps, a *standard working solution* of 2 μg/ml concentration is prepared. The concentration of the standard working solution can be checked by spectrophotometry, using the following formula:

$$E^{1\%}_{1cm} = 1561 \text{ (measured in ethanol at 325 to 326 nm)}$$

From this it follows that in a 1-cm cuvette the standard solution of 2 μg/ml concentration should give an extinction of 0.312. If values other than this are obtained, the new value shall be used for calculating the final results of determination.

(b) *Standard vitamin E solution.* From D,L-α-tocopherol a stock solution of 10 mg/ml concentration is prepared in pretreated ethanol, using measure-

ment according to weight. 1 mg D,L-α-tocopherol = 0.68 IU. The quantity to be measured out is calculated as described in paragraph (a). From the stock solution a *standard working solution* of 10 μg/ml concentration is made by dilution with pretreated ethanol in several steps. The concentration of this working solution can be checked by spectrophotometry:

$E^{1\%}_{1cm}$ = 72–76 (measured in ethanol at 292 nm).

From this it follows that in a 1-cm cuvette the standard solution of 10 μg/ml concentration should give an extinction between 0.72 and 0.76. If values other than this are obtained, the concentration shall be calculated and the new value thus obtained shall be used for calculating the final result of determination.

The standard solutions shall be stored in brown bottles in a refrigerator. Their concentration shall be checked on every day of analysis.

3. *Determination*

The solutions are measured into 15 ml centrifuge tubes with polished glass stoppers.

	Sample	Standard	Blind
Serum (ml)	0.2	—	—
Standard vitamin A working solution (ml)	—	1.0	—
Standard vitamin E working solution (ml)	—	1.0	—
Distilled water with sodium ascorbate (ml)	1.0	1.2	1.2
Ascorbic acid in ethanol (ml)	2.0	—	2.0

(If only vitamin A or only vitamin E is to be measured, the "unnecessary" vitamin solution shall be replaced with its solvent, ascorbic acid in ethanol).

— Plug the tubes and shake them on a test-tube shaker for 15 s. Check the time with a chronometer.

— Add 5 ml petroleum ether.

— Shake the mixture vigorously by hand or on a test-tube shaker. Check the time with a chronometer.

— Centrifuge the mixture at 1500 rpm for 5 min. Store the tubes closed, wrapped in aluminium foil, and keep them away from light and heat.

— One ml volumes of the upper (petroleum ether) phase are used for the measurements. Since in the petroleum ether phase vitamin concentrations remain stable for some days, instrumental measurement can be postponed a day or two if necessary.

4. *Measurement*

Adjust the fluorometer (Hitachi 650-10 S)

	Vitamin A	Vitamin E
Excitation wavelength (nm)	375	295
slit width (nm)	5	2
Emission wavelength (nm)	480	325
slit width (nm)	8	8
Sensitivity	10	1

The 375 nm excitation wavelength and 480 nm emission wavelength are suitable for measuring both vitamin A and vitamin A acetate.

There are two possible ways of measurement:

(a) With the diaphragm closed, the instrument is set to 0. Then the diaphragm is opened and the fluorescence values of the blind control, the standard solution and the test samples are determined.

(b) One ml of the blind control is considered 0 to which the instrument is set, then the fluorescence values of the standard solution and test samples are measured.

One ml volumes of the unknown serum samples and standard solution are measured in the same cuvette. Between the measurements the cuvette is thoroughly rinsed with 2×1 ml petroleum ether.

The most practical way of measuring is when the samples are first assayed for one of the vitamins, then, after setting the instrument, they are assayed for the other vitamin, using 1 ml volumes of the petroleum ether phase.

5. *Calculation*

By the method presented in this paper the quantities of *total vitamin A* and *free vitamin E* are determined (Hansen and Warwick, 1978). About 60% of the free vitamin E is α-tocopherol, and the remainder is β-tocopherol and some other derivative(s). Vitamin A and E concentrations are calculated by the following formula:

— using measurement (a)

$$\text{vitamin A or vitamin E } (\mu g/ml) = (X - B)\frac{C}{(S - B)}$$

— using measurement (b)

$$\text{vitamin A or vitamin E } (\mu g/ml) = \frac{C \cdot X}{S}, \text{ where}$$

X = fluorescence value of the sample
B = fluorescence value of the blind

S = fluorescence value of the standard solution
C = concentration (μg/ml) of the standard working solution.
In the literature the quantity of vitamin A is often expressed in μg/100 ml.

Results

Sensitivity of the method

For vitamin A: 20 μg/100 ml serum
For vitamin E: 0.04 μg/ml serum

Recovery rates

For vitamin A: 87 to 106%
For vitamin E: 89 to 106%

Vitamin concentration and the measured fluorescence were in a linear correlation in the range of 0.2 to 4.0 μg/ml for vitamin A and 0.04 to 10.0 μg/ml for vitamin E.

Sensitivity of the method, recovery rate and linearity were determined in several parallel measurement series, by adding standard solutions of different quantity (reaching the detection limit) and by sample analysis. To avoid lengthiness, these results are not given here in detail.

Applicability of the method was tested by determining the vitamin A and vitamin E levels of the serum of a total of 160 animals of 8 different species. The results are summarized below.

	Number of samples	Vitamin A (μg/100 ml)	Vitamin E (μg/ml)
Poultry	33	28—114	1.6—4.7
Goose	30	20—132	0.6—4.3
Cattle	13	20— 52	1.0—4.9
Swine	14	<20	—0.3
Sheep	18	<20	0.2—1.2
Horse	16	— 48	0.5—1.7
Rabbit	12	31— 71	0.8—1.7

These values do not differ markedly from the so-called normal values published in the literature.

Twenty-one of the 33 poultry serum samples assayed were collected during a carotene-overdosage experiment. The results of vitamin A determination reflected the difference between the control and carotene-treated groups and the carotene dose fed.

Discussion

From the "Materials and methods" section it can be seen that lengthy and careful preparations are needed for the determination. Since large quantities of the necessary solutions can be prepared at a time and only little is needed for a determination, solvents sufficient for several hundred determinations can be obtained by 1 or 2 days' preparatory work.

Fluorometry requires extremely pure solvents. Contaminating substances present in the solutions may fluoresce or, contrarily, absorb some of the vitamins' fluorescence at the wavelength of measurement, thus leading to false results. Investigations into this problem showed that e.g. in vitamin E determination higher values were obtained with pretreated than with untreated ethanol. The distillation of petroleum ether is necessary because the steam pressure of its light distillate fraction may pop out the stopper, thus leading to loss of material.

Accepting the recommendation made by Driskell et al. (1985), to the ethanol used for extraction we added ascorbic acid. This prevents losses due to decomposition of vitamins A and E by ethanol.

To ensure optimum adjustment of the fluorometer the excitation and emission curves of vitamins A and E were determined at different slit widths and sensitivities. The measurement parameters were derived from these data. Several methods use hexane for vitamin extraction. However, in the Raman emission spectrum of hexane, at an excitation of 295 nm there is an emission peak at 325 nm. Since the emission peak of vitamin E is at the same wavelength, for vitamin extraction we used petroleum ether which does not have Raman emission at that wavelength. At an excitation of 295 nm the emission curve of petroleum ether is lower than that of hexane: therefore, its increasing effect on the blind value is less.

The determination itself is very simple, rapid, highly reproducible, gives good recovery rates, is suitable for serial determinations, and is more sensitive than are conventional vitamin determinations. Its drawback is that serum vitamin A concentrations lower than 20 μg/100 ml are not measurable. Thus, severe vitamin A deficiencies of cattle, sheep, horses and swine are not detectable by this method. Attempts were made to lower the detection limit by using five- or tenfold quantities of sample and of extraction solvent and by concentrating the extract. Unfortunately, the results were not interpretable, showed large deviations and did not correlate with the vitamin quantities added. Since vitamin E has a much broader measurement range, its deficiency can be monitored accurately.

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POSSIBLE APPLICATIONS OF THE "MANUFLEX" EXTERNAL FIXING INSTRUMENT IN SMALL ANIMAL SURGERY

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An external fixing instrument (minifixateur externe) recently developed for use in humans and marketed by the name of "MANUFLEX", its accessories, possible applications and fields of indication are described. The apparatus has been patented in Hungary and an international patent has been applied for.

In the second part of the paper the application of the external fixateur for treating bone fractures of dogs is reported, together with the practical experience gained with it so far.

As the clinical trials gave favourable results and since the instrument is available for veterinary practitioners at a reasonable price, MANUFLEX may become a gap-filling apparatus in the instrumentarium of small animal surgery.

Keywords: MANUFLEX, external fixation, bone fractures, small animal surgery.

In recent years, parallel to the increase in the number and value of small animals, the need has arisen for an animal health care of higher standard.

In the field of small animal traumatology this reasonable need can only partially be satisfied. Although the treatment of extremital bone fractures of traumatic origin has become a routine task first of all in the veterinary practice of big cities (Berge and Westheues, 1969; Brinker et al., 1984; Fellner and Szokolóczy, 1976; Tóth and Graf, 1983), for lack of proper technical conditions treatment is often restricted to conservative procedures (e.g. different splints) by which the desired effect is not always achieved. Apart from a few privileged institutes, the instrumentarium required for surgical treatment of bone fractures is unattainable and far too expensive for veterinarians.

An inexpensive external fixateur originally developed for use in human hand surgery has recently become commercially available for small animal practitioners.

In the first part of the present paper the external fixateur and its accessory instrumentarium, while in the second part initial experience gained with the instrument in dogs are described.

Malgaigne (cit. Hoffmann, 1939) was the first to report on the use of an external fixation device in 1853. At the end of the last century, Berenger

and Faraud (cit. Hoffmann, 1939) fixed bone screws drilled into the broken ends proximal and distal to the fracture line to an external wooden frame.

Parkhill (1897) constructed an external frame made of metal. The immediate ancestor of the presently used external fixateurs was developed by Haynes in 1939. The elaboration of compression possibilities is linked with the name of Judet, while Cuendet constructed an aiming device for the instrument. The procedure and instrument recommended by the AO Society (Arbeitsgemeinschaft für Osteosynthesefragen) were elaborated by Müller et al. (1970).

By now several different external fixateurs have become known in human bone surgery (Hoffmann, 1939, 1954; Ilisarov, 1976; Wagner, 1977).

Since the first description of the minifixateur several types (Kesler-Anderson, AO, Tower, Matev, etc.) have become commercially available. In Hungary Hoffmann's minifixateur (Hoffmann, 1939, 1954) is used most widely.

After Hoffmann's model, Becker (1974) constructed an external fixateur applicable in small animal practice. Bush (1977) used an external fixing instrument for treatment of bone fracture in birds. Gahring (1980) described a three-dimensional method. Hurof and Seer (1968), Latimer et al. (1977), Meynard and Goudichaud (1977), Peters and Wildenberg (1981) and Goudichaud et al. (1982) recommended external fixation with acrylate resin. However, no consistently good procedure has been reported yet, and at present no external fixing device is manufactured for use in animals.

As the fixing instruments manufactured abroad for use in human medicine are hard to come by and expensive, their use in veterinary medicine is out of the question. For lack of suitable instruments, in small animals external bone fixation is accomplished using a combination of Kirschner wires and acrylate cement. The drawbacks of this method are that stability is not perfect, after-corrections, distraction and compression are not possible, the polymerization time is rather long, and hardening is accompanied by substantial heat release. This heat is transmitted to the bone; therefore, the frame must be cooled.

Classical indication fields of external fixateurs

External fixation devices had originally been constructed for the treatment of heavily contaminated, splintered, compound fractures accompanied by severe injuries of the soft tissues and, occasionally, by loss of the soft parts and bones. Namely, in such cases internal fixation (with pinning, wiring, plates and screws) is contraindicated because of the risk of septic complications, and plaster-bandage is usually unsuitable for stable fixation and air-dressing at the same time. This has resulted in a gradual widening of the application of

external bone fixation devices. The fields of indication now include *(i)* open fractures; *(ii)* bone and articular injuries combined with septic complications; *(iii)* prolonged bone healing, septic pseudoarthrosis; *(iv)* arthrodesis; *(v)* osteotomy, osteoplasty; *(vi)* treatment of conditions developing after unsuccessful internal fixation attempts; and *(vii)* neoplastic surgery and restorative surgery.

The "MANUFLEX" disposable external fixateur. Instrumentarium (Fig. 1)

The "MANUFLEX" external fixateur consists of small external fixation sticks and Kirschner wires (1.5 to 2 mm in diameter) which fit into the holes of the sticks (Fig. 2). In addition, only three pairs of pliers (Fig. 3) are needed. With the modelling pliers the sticks made of flexible metal alloy can be bent to a shape corresponding to that of the limb. With the cutting pliers the sticks and wires are cut to the desired size. The presser (Fig. 4) establishes a firm link between the wires and sticks serving as the external frame.

Frame configurations of varying shape and strength can be established by altering the number of Kirschner wires and the direction of boring.

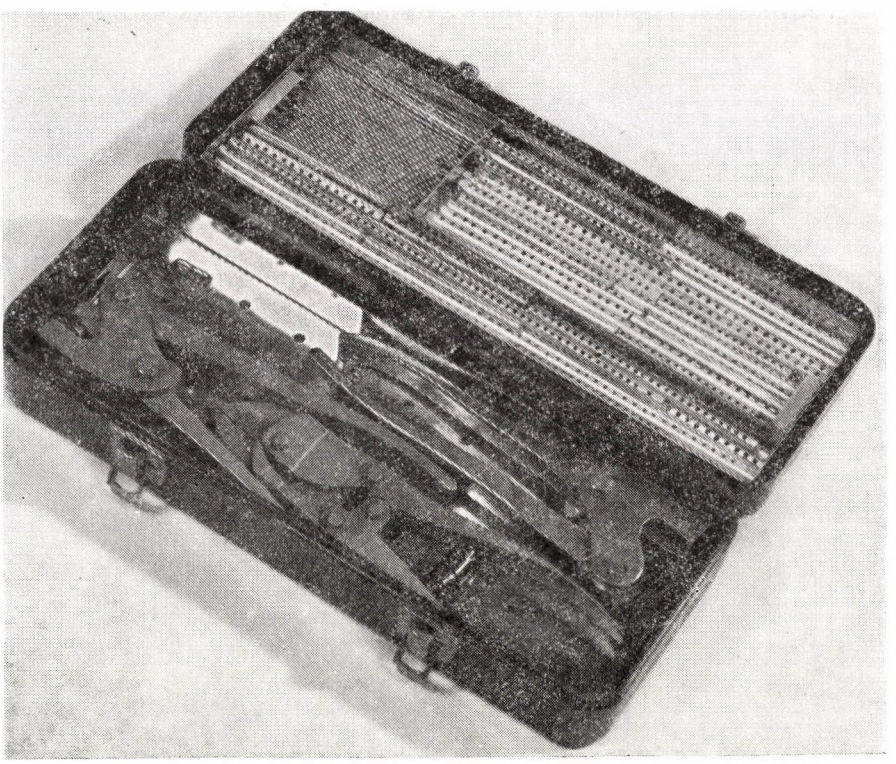


Fig. 1. The MANUFLEX instrumentarium

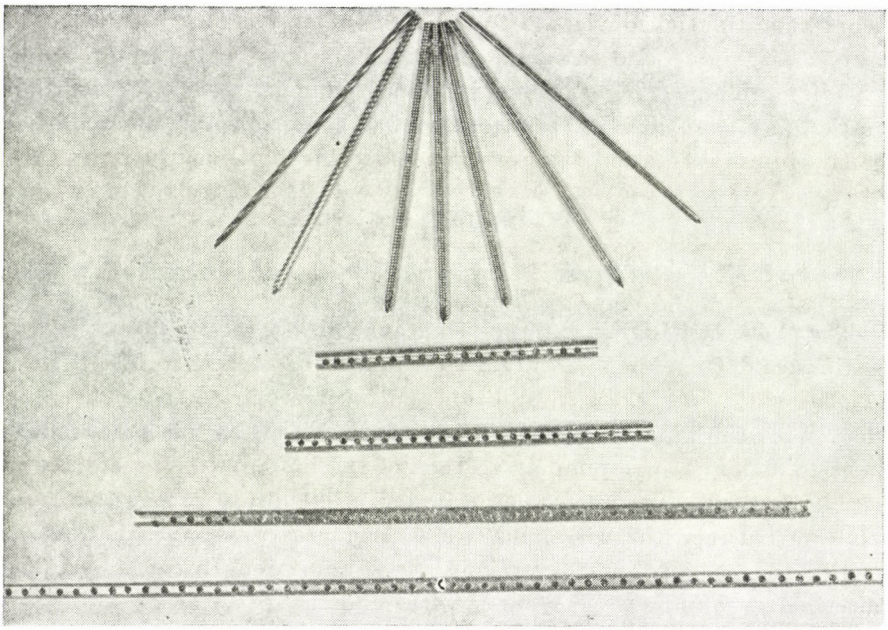


Fig. 2. MANUFLEX sticks and Kirschner wires belonging to the external fixateur

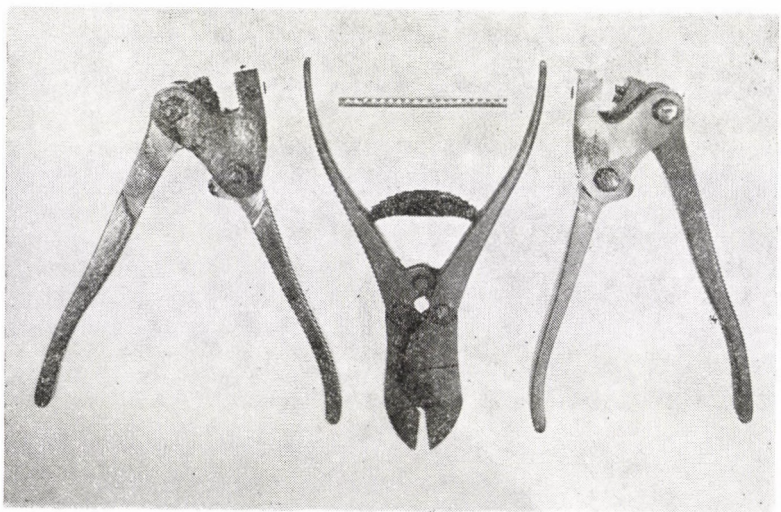


Fig. 3. Pliers belonging to the instrumentarium: modelling pliers, cutting pliers and presser

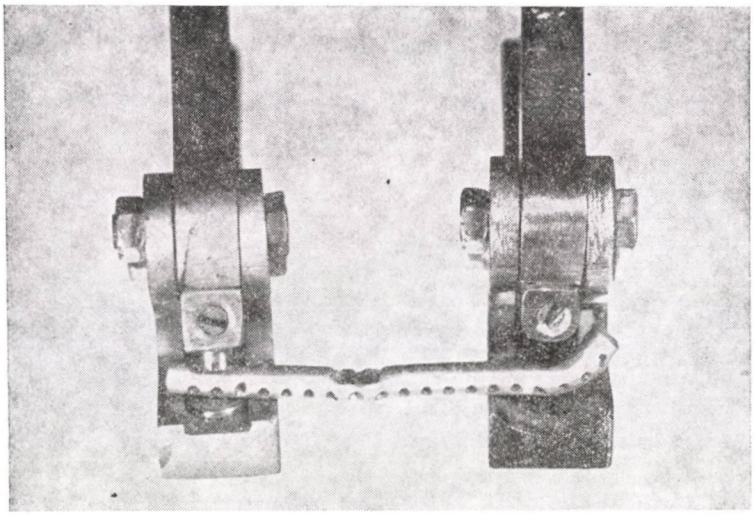


Fig. 4. Use of the presser and modelling pliers

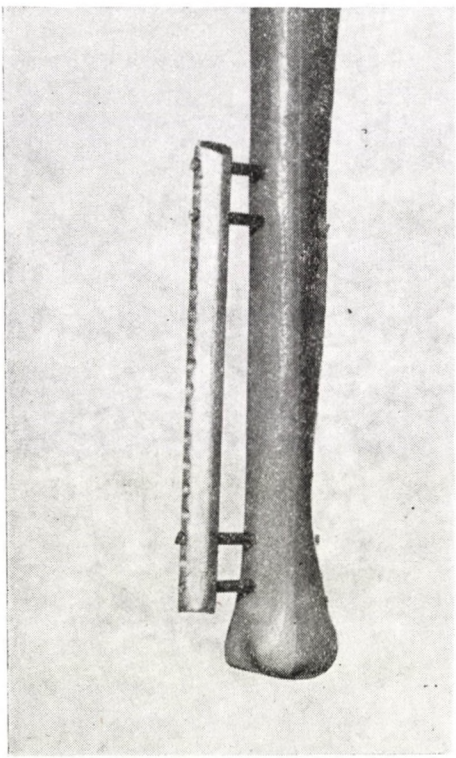


Fig. 5. Half frame configuration

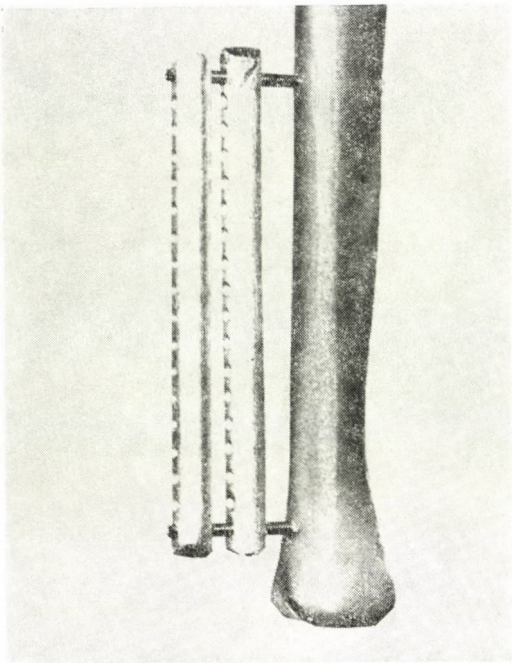


Fig. 6. Strengthened half frame

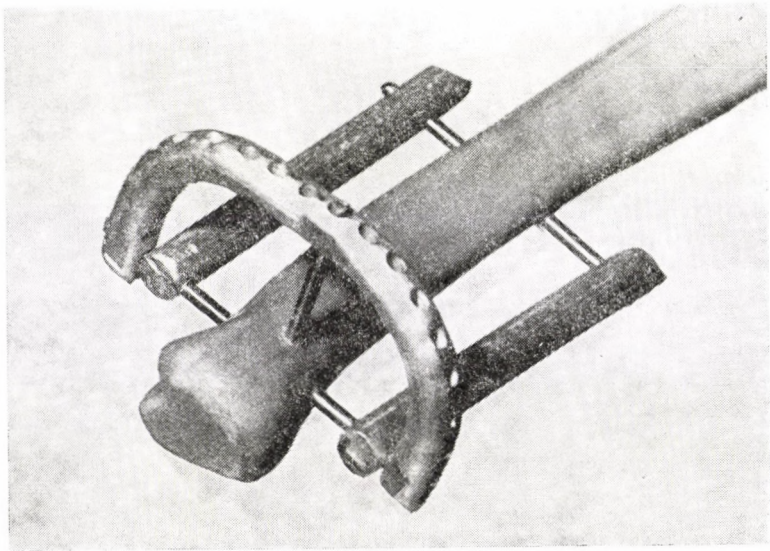


Fig. 7. Triangular or hemicircular frame

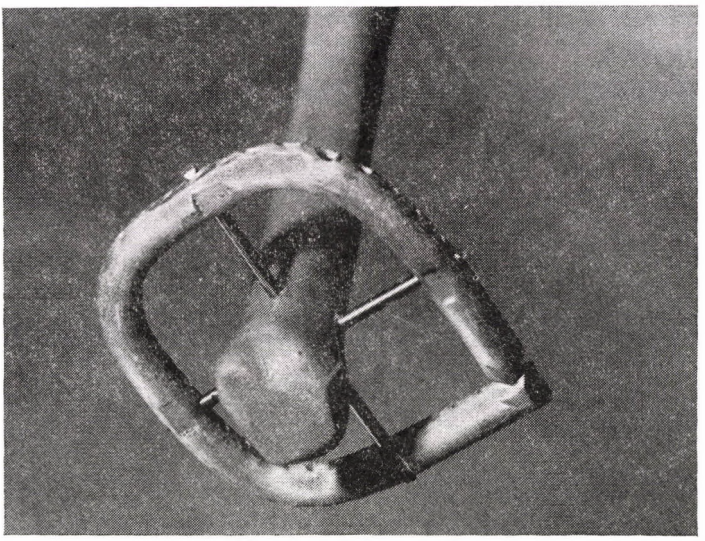


Fig. 8. Circular frame

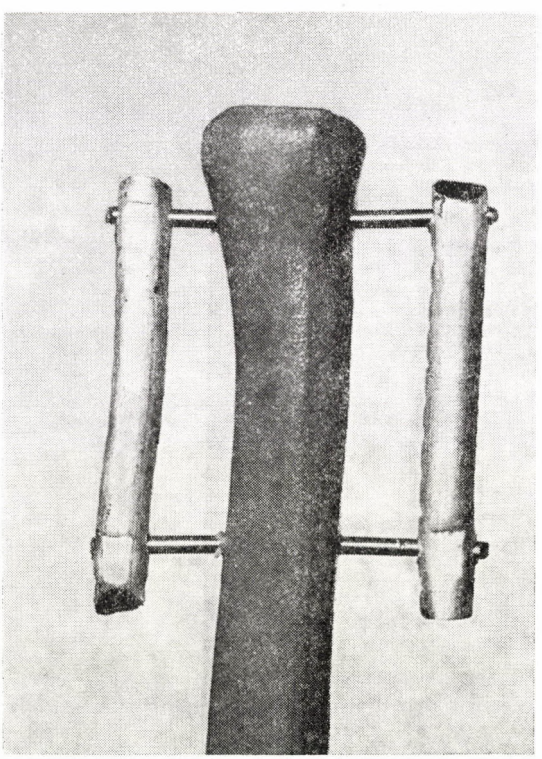


Fig. 9. Whole frame

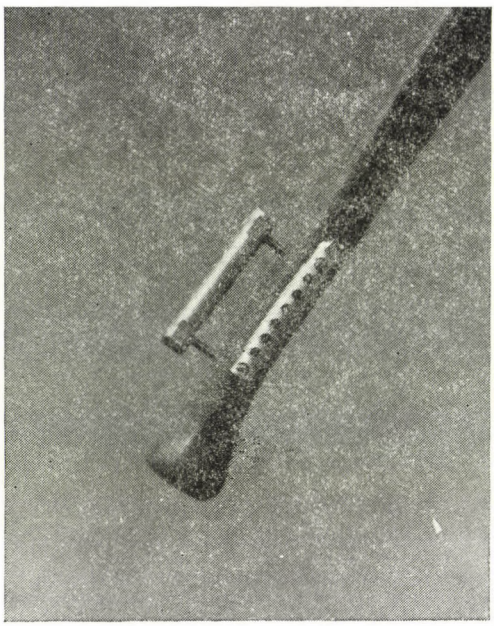


Fig. 10. Double half frame

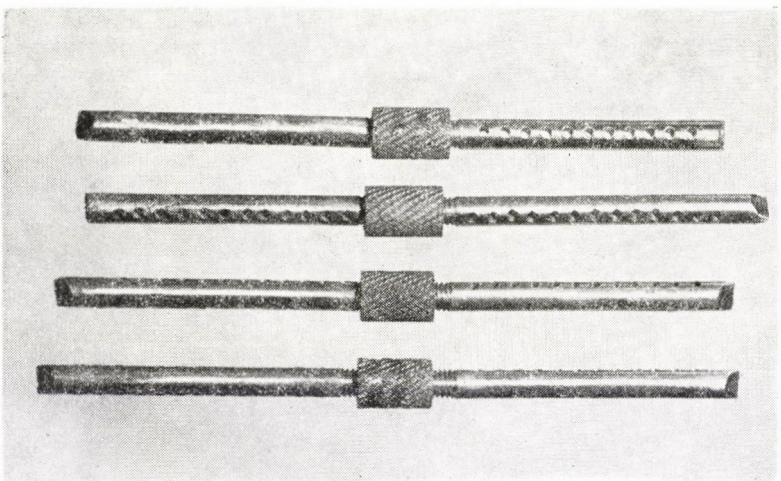


Fig. 11. Disposable compression-distraction adapter

Half frame (Fig. 5)

Two or three wires are drilled into each broken end from the side or in the sagittal plane in a way that the wire ends become settled in the cortex on the opposite side. To the protruding wire ends one MANUFLEX stick or, in

order to strengthen the frame, two MANUFLEX sticks are fixed (Fig. 6). The advantage of this fixation procedure is that there are fewer perforations in the skin and the risk of infection is lower. However, it is less stable and can be recommended for use primarily in animals of small body size, weighing a few kilograms.

Whole frame (Fig. 9)

The wires drilled into the bone are led out on the other end and are fixed with MANUFLEX sticks on both sides. This gives a fixation which is more stable and has greater supporting power than the half frame.

Stability can be increased and rotation prevented by using the following configurations:

Double half frame (Fig. 10)

Two half frames perpendicular to each other (usually situated in the horizontal and sagittal planes, respectively). Stability is further increased by connecting the ends of the half frames, which thus form a quarter circle.

Triangular of hemicircular frame (Fig. 7)

This frame consists of three half frames: two horizontal half frames and a sagittal one. The ends of the half frames can be connected with straight or bent sticks: thus a triangular or hemicircular configuration is obtained.

Circular frame (Fig. 8).

The circular frame consists of four half frames or two whole frames perpendicular to each other. The ends are connected by a circular stick.

MANUFLEX external minifixateurs should be considered disposable since they suffer lasting deformities during use. Compression and distraction can be accomplished also with this instrument if it is supplemented with a disposable compression adapter (Fig. 11).

In human surgery MANUFLEX can be used for treating fractures of small tubular bones, first of all in hand, leg and paediatric surgery. An additional indication field is maxillofacial surgery.

Materials, methods and results

The "MANUFLEX" external fixateur was first tried out on 5 clinically healthy, 1 to 5 years old, male and female dogs of 15 to 35 kg body mass. The dogs were anaesthetized with 0.3 mg/kg/body mass propionylpromazine (Combelen, Bayer, Leverkusen, FRG) and 2 mg/kg body mass methadone hydrochloride (Depridol), and their tibia and fibula were broken on one side. In two dogs closed, whereas in the other three animals open, fractures were produced. The fractures were always accompanied by severe injury of the soft parts. The broken ends were fixed with a MANUFLEX frame as follows.

The dogs' shank was shaved and the operative field was disinfected and isolated *lege artis*. A 2-mm-thick Kirschner wire was drilled into the proximal broken end. Subsequently the broken ends were reduced, and a MANUFLEX stick cut to size was fitted to the wire. A second wire was drilled into the distal broken end through a hole of the stick. This was followed by other wires: as required, 1 or 2 additional wires were applied on both ends of the fracture. The protruding (superfluous) ends of the wires were cut off and the stick was compressed with the presser. Thus a firm link was established between the stick and the wires. At the end of the operation the surfaces of contact between the skin and the spit were covered with a special disinfectant solution, which formed a thin, adhesive film. This solution was applied onto the surface daily.

The whole operation lasted only 15 to 20 minutes. The dogs regained consciousness without any complication and could almost immediately bear weight on the operated limb.

In the postoperative stage the dogs were kept in cages but were walked on leash daily. No analgetics or antibiotics were used. The external fixateur was removed from all five dogs 8 weeks after the operation. The postoperative stage was uncomplicated.

The dogs used their operated limb without any sign of motor disturbances. Callus formation was already well visible in radiographs taken before the removal of the external fixateur. Periosteal callus was not excessive, indicating that the fixation was stable. The fracture line was scarcely visible. The favourable changes of bone trabecules also called attention to the benefits of this stable but dynamic fixation.

After trials on experimental animals the instrument was used on a wide variety of clinical patients, in a total of 18 cases: in 12 dogs tibial and fibular fractures and in 6 animals fractures of the radius and ulna were treated. Four out of the 18 were open fractures. Seven fractures were accompanied by severe injury of the soft tisuses. The patients were 1 to 10 years old dogs of various breeds. The fixateur was put on within 48 h after the trauma.

Depending on the fracture to be treated, half frames (Figs 12, 13 and 14), whole frames (Figs 15, 16, 17 and 18), or double half frames in so-called "quarter circle" configuration (Figs 19, 20 and 21) were used.

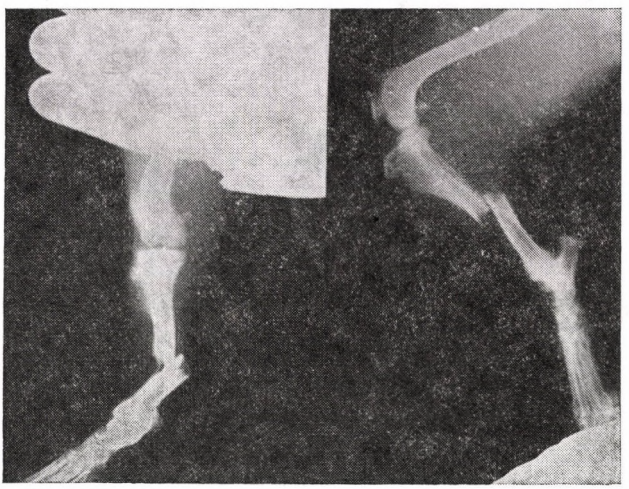


Fig. 12. Radiograph of a tibial fracture from two sides

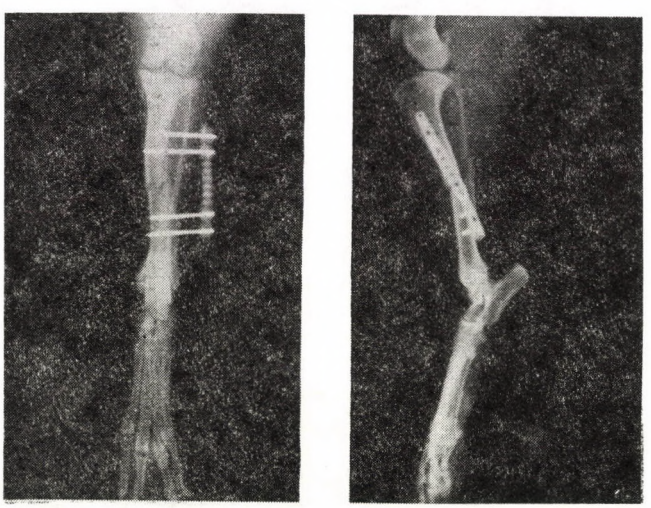


Fig. 13. Fixation of the broken ends after reduction

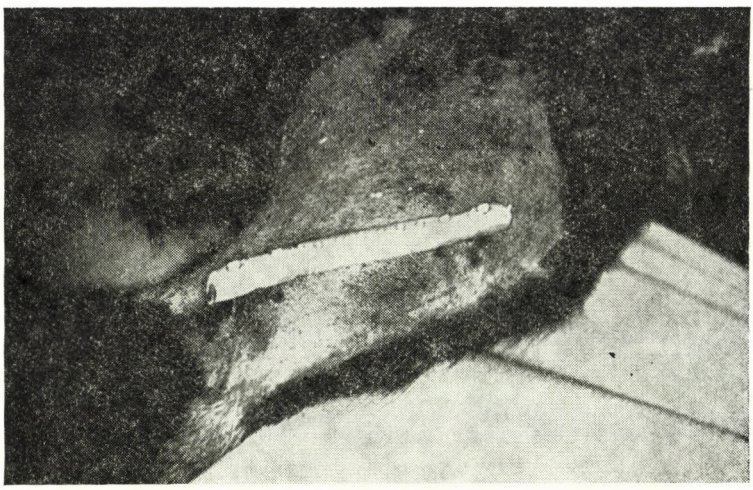


Fig. 14. Half frame fixed in place

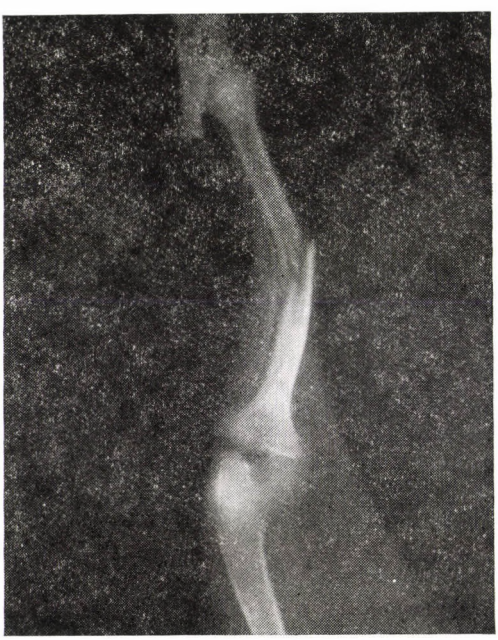
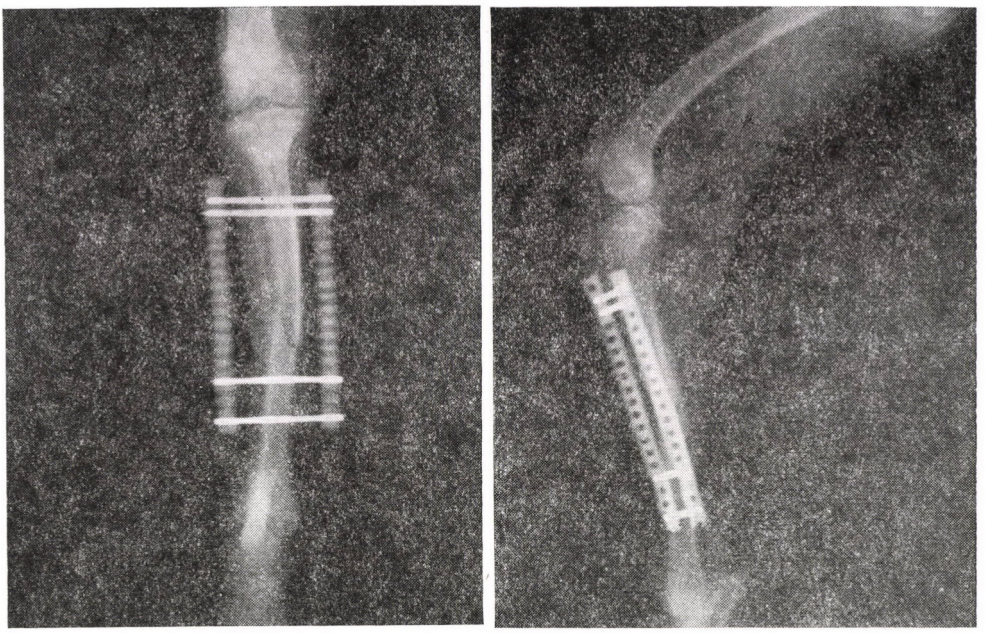


Fig. 15. Radiograph of a willow fracture of the tibia



Figs 16 and 17. Radiograph from two sides, taken after surgery

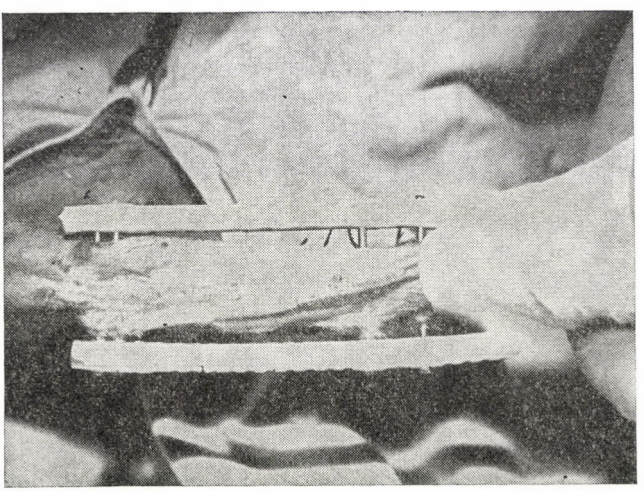


Fig. 18. Whole frame fixed in place

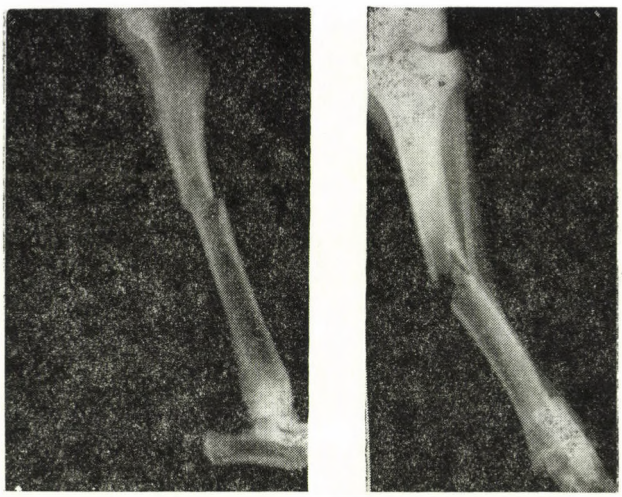


Fig. 19. Radiograph of a tibial fracture from two sides

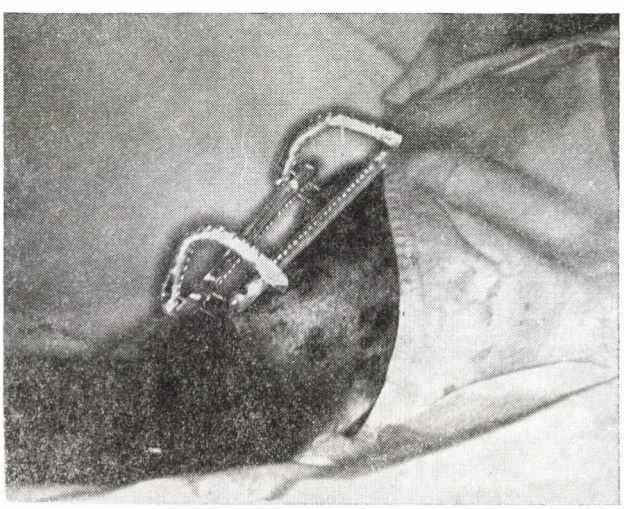


Fig. 20. Double half frame with quarter-circle fixation (postoperative stage)

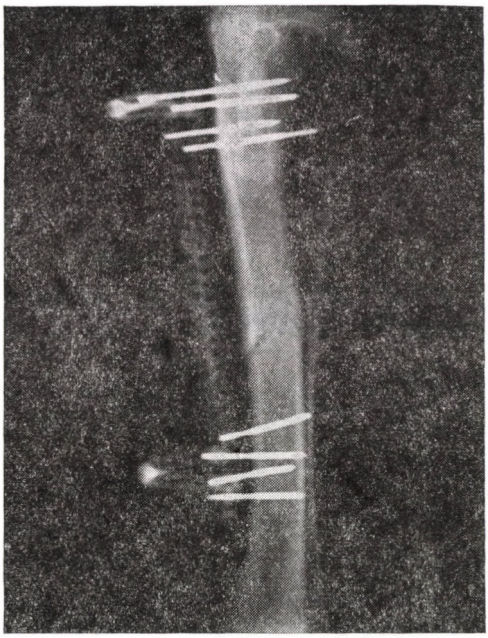


Fig. 21. Healed tibial fracture before removal of the external fixateur

The patients were let home immediately after the operation. The owners were recommended to walk the dogs on leash over a period of 3 weeks. Recovery was checked weekly or, if necessary, even more frequently. Dogs with open fractures and severe soft tissue injuries received antibiotics. Recovery was uncomplicated. In two dogs with severe soft tissue injuries a considerable swelling occurred which subsided by postoperative day 4 to 5 and subsequently disappeared.

Around 8 out of the 112 Kirschner wires there was slight secretion. Deep infection or osteomyelitis did not occur.

Discussion

The experience gained with MANUFLEX on the 5 experimental dogs and 18 clinical patients is favourable. The MANUFLEX external fixing instrument is excellent for routine surgical treatment of bone fractures of dogs and other animals of small body size. Its use is easy to learn: operators with proper anatomical knowledge can place it on easily. The variability of metal frame configurations allows the surgeon to treat different fractures successfully. The MANUFLEX external fixing instrument is light, it does not ob-

struct the movement of injured animals and permits proper treatment of injuries of the soft tissues. It gives stability and rest for bone healing and permits active movement at an early time. The animals tolerate the external fixateur easily.

In our experience, it is expedient to carry out the operation within a short time (possibly within 24 to 48 h) after the trauma, since the success of the operation greatly depends on reductibility of the broken ends.

Possible swelling of the limb should be considered when the MANUFLEX external fixateur is put on. Therefore, the sticks should be placed at sufficient distance from the skin.

In addition to its above-listed classical human indication fields, the external fixateur can be used in small animal surgery. It is recommended for treating fractures of the radius, ulna, tibia, fibula, metacarpals, phalanges and mandible in cases when, for lack of technical and/or financial conditions, their firm internal fixation is impossible and conservative treatment does not seem promising.

The apparatus is inexpensive and its use is easy to learn. The time of the intervention is 15 to 20 min and the material costs of the operation are negligible. Therefore, the use of MANUFLEX would considerably improve the standard of traumatological care of small animals.

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COLONIC ANASTOMOSIS IN CALVES: AN EXPERIMENTAL STUDY

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Colonic anastomosis was conducted in 12 calves divided equally into four groups. Four suture patterns, viz. single inversion (continuous Connell), double inversion (two rows of continuous Cushing), single eversion (continuous everted mattress), double eversion (single eversion reinforced with simple continuous) were used. In each animal, anastomosis of the same technique was performed at four places. Each technique was evaluated in terms of clinical and gross observations, lumen stenosis and bursting pressure. Observations were made on the 7th, 14th and 28th day after anastomosis.

None of the anastomotic techniques showed any untoward incident during the 28 days of postoperative study. Stricture formation at the site of anastomosis was minimal with the single inversion technique whereas it was maximum with the single eversion technique. Pressure for disruption of intestinal segments was higher after inversion than eversion techniques. It was concluded that the one-layer inversion anastomosis technique proved to be the best among the four techniques because *(i)* of its reduced incidence of adhesions; *(ii)* it maintains adequate lumen diameter; *(iii)* it withstands maximum pressure. The double layer inversion technique was conceded to be the second best choice for colonic anastomosis followed by the double eversion and single eversion techniques.

Keywords: Colonic anastomosis, calf, suture patterns, experimental study.

Various alterations of colon, viz. obstruction, segmental aplasia, diverticulum, abscessation, adhesions, neoplasia, volvulus and incarceration require resection and anastomosis of the affected portion of the colon (Horney and Wallace, 1984). Perusal of literature indicated intestinal leakage, wound dehiscence and stenosis as perplexing complications of different entero-anastomotic techniques. Therefore, every attempt is directed to minimize these complications besides evolving a simple suture pattern which is less time consuming and results in minimal fibrosis with first intention healing. In bovines, because of the larger diameter of lumen, poorer vascularization, presence of faecal load with a higher number of microorganisms in the colon as compared to the small bowel, a particular suture pattern is yet to be evaluated for the colonic apposition.

Keeping in view the significance of the problem and paucity of work available on this aspect, an experimental model of four different techniques for colonic anastomosis was developed and results were evaluated in terms of clinical and gross observations, bursting pressure and luminal stenosis of colonic anastomosis site.

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Materials and methods

The present study was conducted on twelve healthy cow calves of 1 to 1 1/2 years of age. The calves were dewormed one week prior to the experiment and were kept under similar managemental and feeding conditions throughout the experiment. These animals were divided equally into two main groups. The animals were further sub-grouped (three in each) according to the intended entero-anastomosis technique.

The animals were kept off feed and water for 24 h before surgery. They were controlled in left recumbency under sedation with 6% chloral hydrate, and the right paralumbar fossa was prepared for aseptic surgery. The abdomen was entered through a linear incision under local infiltration of 2% lignocaine hydrochloride. A colon segment was exteriorized and covered using sponges soaked in saline solution. The contents of the loop were digitally massaged and two straight intestinal forceps were placed, i.e., one on either side of the proposed surgical site. The intestine was then sharply transected without incising the arcade vessels. Anastomosis was performed with 3/0 silk using inversion or eversion suture patterns (Table I). The same type of anastomotic technique was repeated at three more sites distal to the previous one at approximate intervals of 30 cm. The exteriorized colon sections were rinsed with warm saline solution and replaced. The abdominal incision was then closed in the routine manner.

The calves were allowed to take food (soft green) and water *ad libitum* 12 h after surgery. Postoperative medication comprised parenteral administration of oxytetracycline, B-complex and analgin. Antiseptic dressing of the skin wound was performed daily.

Table I

Details of colonic anastomosis

Group		Anastomotic technique	Suture pattern	No. of anastomoses
I		Inversion		
(6)	(i)	Single layer (3)	Continuous Connell's as described by Larson and Bellenger, 1974	12
	(ii)	Double layer (3)	Continuous Cushing as described by Reinertson, 1976	12
II		Eversion		
(6)	(i)	Single layer (3)	Continuous mattress as described by Kumar and Singh, 1971	12
	(ii)	Double layer (3)	Continuous mattress followed by simple continuous as described by Kumar and Singh, 1971	12

In parentheses: number of animals

Evaluation of clinical status included monitoring of rectal temperature, heart rate, respiratory rate, defaecation and general condition of the animal throughout the period of study.

The animals subjected to the same anastomotic technique (Table I) were euthanatized by carotid artery bleeding on the 7th, 14th and 28th day after surgery, respectively. The intestinal mass with the anastomotic sites was removed and kept in normal saline solution for the studies.

Gross observations included information on the *(i)* condition of omentum and mesentery; *(ii)* adhesions of the anastomotic site with the adjacent tissues; *(iii)* leakage of ingesta, presence of pus or perianastomotic abscess; *(iv)* any other intraabdominal lesions such as haematoma, haemoperitoneum or peritonitis.

Luminal stenosis

Colon pieces of 15 cm length, with the anastomotic site at the centre, were resected. The cut ends were tied tightly and barium sulphate suspension was infused into the lumen with a syringe until the specimen expanded. The radiographs were made at 55 KVP, 10 MAS and 90 cm FFD. The percentage of luminal stenosis was calculated by the formula $100\left(1 - \frac{2A}{B + C}\right)$, where "A" stands for the width of the suture line, and "B" and "C" for the width measured 2 cm proximal and distal to the suture line, respectively, on the radiograph.

Bursting pressure

After taking barium radiographs, one end of the test intestinal segments was untied and barium sulphate was washed away. The open end was connected to a mercury manometer through a polythene tube and a three way cannula (Fig. 1). The test segment in the collapsed state was immersed in water and then gradually inflated by injecting air with a syringe. The bursting pressure was noted on the mercury manometer the moment an air leak appeared or the intestinal test segment burst near the anastomotic site.

Control studies

Bursting pressure studies were conducted on a normal colon loop taken from the same animal which was subjected to anastomoses. Each experimental animal served as its own control and thereby control studies on a separate group of animals were avoided.

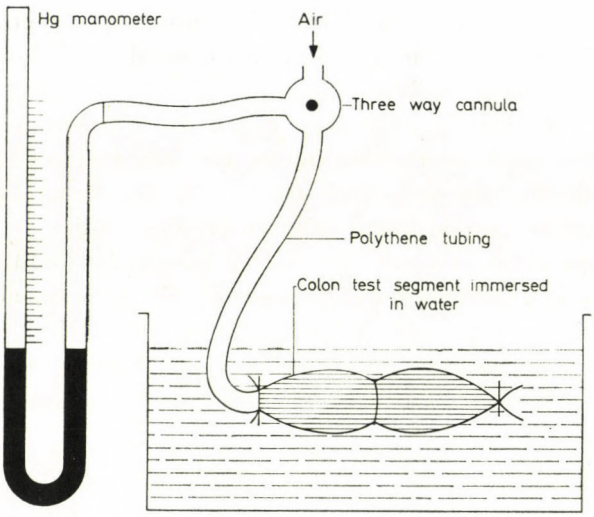

Fig. 1. Bursting pressure device

Results

Clinical and gross observations

All the calves passed faeces within 24 h after surgery. The faeces was of normal consistency though one animal from the double eversion group showed blood in the faeces on the third post-surgical day. None of the animals showed signs of intestinal obstruction and took feed and water normally during the period of observations. A rise in body temperature was observed on the 3rd or 4th postoperative day in all animals. In animals of the eversion group this continued even up to the 10th day. The heart and respiratory rates were within the normal range in all animals throughout the period of observation.

Gross lesions observed at the time of sacrifice included an inflamed omental suture line in all the animals at 7 days after the anastomoses. Both the omental layers, though adhered to each other, were separable by slight pulling at 14 days. The omental layers were found almost normal at 28 days after surgery.

The sites of anastomoses adhered to the adjacent loops of colon, omentum and mesentery. The postoperative adhesions observed in animals of the different groups were graded by severity from — to ++++ (Table II).

At 7 days after surgery, adhesions were severe with the eversion technique and could be separated only by scissors. Adhesions were scanty in single inversion, while in the double inversion technique they were slightly more numerous and could be broken by fingertips. Maximum adhesions were noted with the omenta, mesentery and adjacent loops of colon with the single eversion

Table II

Postoperative adhesions

Technique	Days after surgery		
	7	14	28
Single layer inversion	+	−	−
Double layer inversion	++	+	+
Single layer eversion	+++	++++	+++
Double layer eversion	+++	+++	+++

(−) negligible adhesions
(++++) maximum adhesions

technique at 14 days. These adhesions could only be severed by scissors. Adhesions were negligible in single inversion but scarce and fibrotic in double inversion technique at that time. By day 28 the eversion technique had elicited fibrotic adhesions mainly with the adjacent loops of colon. In animals operated on by inversion technique, fibrotic thread-like and easily separable adhesions were seen only after the double inversion technique.

Fibrous tissue reaction was observed grossly in animals of all groups except in the case of the single layer inversion technique. When breaking the adhesions, caseous material was infrequently noted in eversion technique on the 7th and 14th day after surgery. The presence of inspissated pus between two suture lines of the double layer inversion technique was observed at 28 days after anastomosis.

Bursting pressure (mm Hg)

Bursting pressure studies following different anastomotic techniques on colon (Table III) showed an increase in bursting pressure with time. A steep increase was noticed from day 7 up to day 14 after surgery irrespective of the technique used. However, a substantial increase was observed with the single layer technique, but not with the double layer techniques, from day 14 up to day 28 (Fig. 2). The rupture of colon took place adjacent to the suture line on day 7 with all techniques except the single layer inversion where it was noticed just away from the suture line. At subsequent intervals rupture was observed away from the anastomotic site in all the techniques.

Luminal stenosis

The degree of stenosis in different colonic anastomotic techniques (Table IV and Figs 3, 4, 5, 6) showed that stricture at the anastomotic site decreased and increased in inverting and everting techniques, respectively, with time.

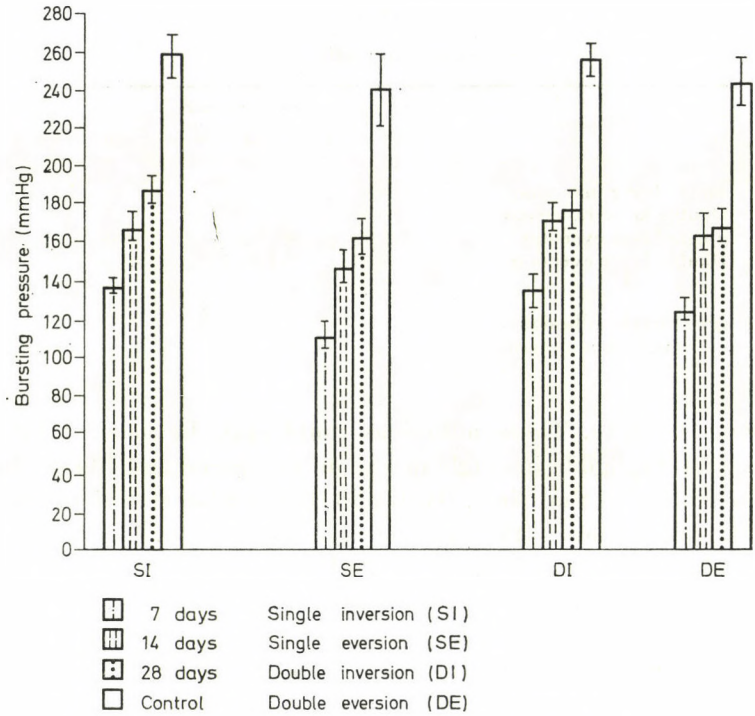

Fig. 2. Techniques of colonic anastomosis

Table III

Bursting pressure (mm Hg) of colon segments following different anastomotic techniques in cow calves (mean ± SD)

Technique	Control	Days after surgery		
		7	14	28
Single inversion	257.50±23.57	137.00±5.77	166.00±15.92	186.25±16.42
Single eversion	239.50±36.38	111.50±11.59	144.50±19.28	162.50±19.82
Double inversion	255.50±17.46	134.50±17.31	169.50±12.48	175.00±27.20
Double eversion	242.50±27.19	124.00±10.95	162.50±15.86	165.00±15.70

Table IV

Luminal stenosis (percentage constriction on the average) at the anastomotic site

Technique	Days after surgery		
	7	14	28
Single inversion	50.00%	32.39%	27.02%
Double inversion	65.11%	55.50%	57.50%
Single eversion	50.00%	59.37%	67.90%
Double eversion	38.23%	37.00%	57.50%

On day 7, stenosis in the bowel at the suture line was minimum in double eversion followed by the single eversion and inversion, and double inversion technique. Maximum lumen of the intestinal segment at the anastomotic site was observed in single inversion followed by double eversion, double inversion and single eversion technique on day 14 of observation. Studies on the 28th post-surgical day showed minimal intestinal stricture formation in single inversion followed by double eversion and inversion, and single eversion.

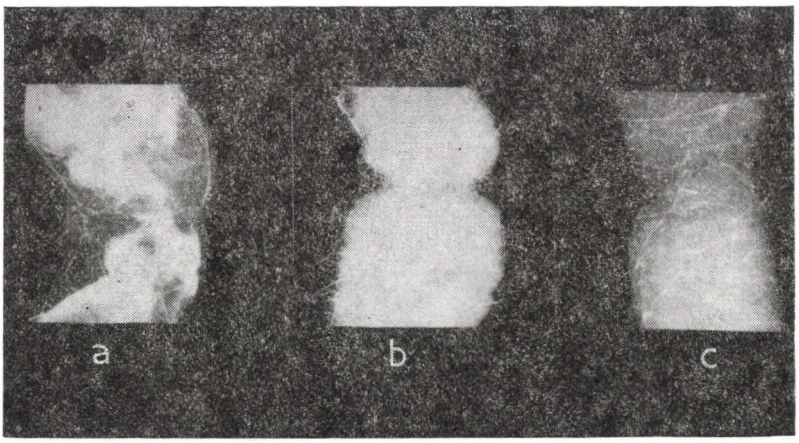

Fig. 3. Barium radiographs of anastomosed colon (single inversion technique) made at day 7 (a), 14 (b) and 28 (c)

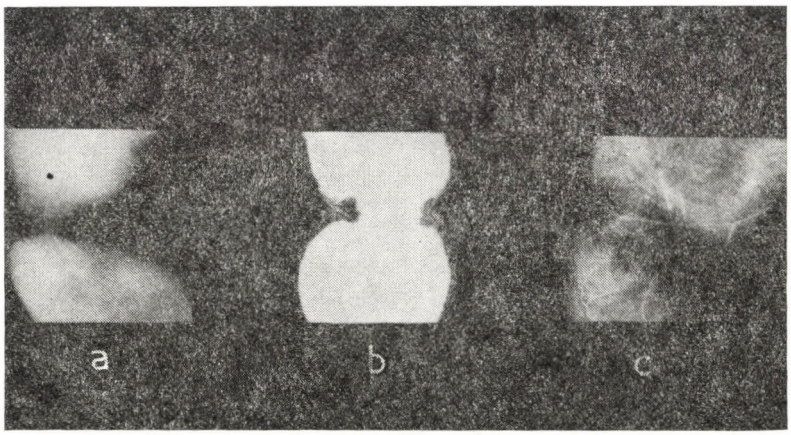

Fig. 4. Barium radiographs of anastomosed colon (double inversion technique) made at day 7 (a), 14 (b) and 28 (c)

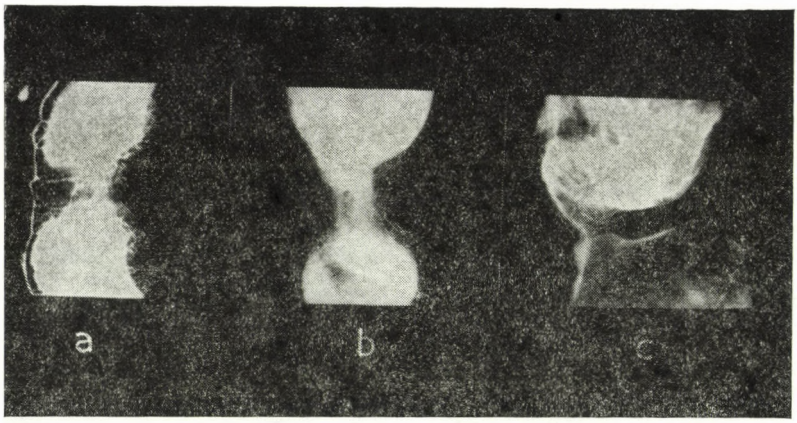

Fig. 5. Barium radiographs of anastomosed colon (single eversion technique) made at day 7 (a), 14 (b) and 28 (c)

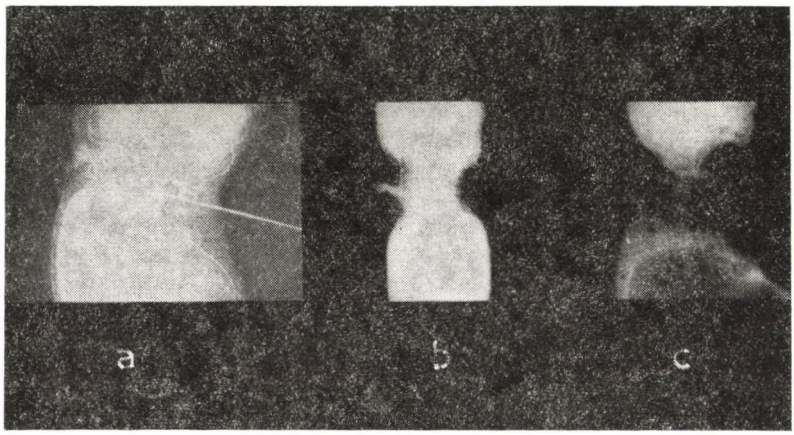

Fig. 6. Barium radiographs of anastomosed colon (double eversion technique) made at day 7 (a), 14 (b) and 28 (c)

Discussion

Clinical observations of all the calves led one to assume the practical possibility of all the anastomotic techniques on the colon of ruminants. However, the gross findings present a different picture in the present investigation.

Rise in body temperature noted on days 3–4 after surgery suggested leakage at the anastomotic site and subsequent peritoneal infection. Johnson et al. (1983) suggested that reduced strength of the gut wall during the first three days might be responsible for the leakage. However, the body tempera-

ture of animals with everting anastomoses continued to rise beyond the 10th post-surgical day due to the presence of bacteria-ridden crypts as a constant source of peritoneal infection (Ravitch et al., 1967; Reinertson, 1976; Singh et al., 1979*a* and 1985).

Unlike extensive adhesions observed in eversion techniques, negligible adhesions were noted in the single inversion technique which could be explained in terms of good apposition/alignment of the severed ends of intestines (Dean and Robertson, 1985), and absence of suture material from the exposed peritoneal surface (Dean et al., 1985). On the contrary, the presence of more adhesions may be attributed to the extensive trauma of the gut wall (Sharma et al., 1976) as well as to a more expressed inflammatory response (Orr, 1969), particularly in the areas of bacterial contamination (Peacock, 1984). These adhesions, however, receded with the passage of time, owing to the development of fibrosis (Singh et al., 1976) and/or shrinkage of the adhesions (Abramowitz and Butcher, 1971). Abscess formation followed by leakage or wound disruption was observed more frequently in eversion techniques. This can be explained by the presence of infection at the wound site during the first few days after surgery representing lag phase (Wise et al., 1975; Hermann et al., 1964; Hawley et al., 1970). Faecal loading may be a factor contributing to leakage (Smith et al., 1983) and subsequent peribowel abscessation. However, no such leakage at the anastomotic site could be observed with the single layer inversion technique due to better apposition and healing of intestinal cut ends (Bronwell et al., 1967). However, the presence of pus in double layer inversion at 28 days after anastomosis was indicative of leakage and setting of focal infection during the early postoperative days.

The inversion technique was invariably stronger than the eversion technique at all times of observation which was evident from the increase in bursting pressure. Maximum bursting pressure observed between postoperative day 7 and 14 in the present study may be attributed to synthesis of new collagen during this period (Herrmann et al., 1964; Cronin et al., 1968; Bone et al., 1983). Conversely, the decrease in bursting pressure in eversion technique may be ascribed to microabscessation, infection, more exposed mucosa, increased adhesions and lesser collagen synthesis (Leob, 1967; Irvin and Coligher, 1973; Hawley, 1970). Moreover, more injury to colon wall occurred when the two-layer technique was used and this increased the collagenase activity in the gut (Hawley et al., 1970). It is believed by Madsen (1953) that excessive tissue reaction around the sutures resulted in a gross weakening of the surrounding tissues. This probably explains why there was lower bursting pressure on postoperative day 28 in the double layer techniques as compared to the single inversion technique.

Luminal stenosis of bowel at all anastomotic sites was more expressed in the eversion technique as compared to the inversion technique. This could

be explained by adhesion formation after eversion. Adhesions at the anastomotic site obstruct the expansion of bowel and shrink with time (Singh et al., 1985) thereby reducing the lumen of bowel at the suture line. In inverting techniques, sloughing of the internal cuff (Reinertson, 1976), reduced oedema of the bowel and gradually decreased primary inflammatory response (Getzen et al., 1966) might be the causes of decreased stenosis of the anastomosed segment. However, after double inversion a thickened mural fibrotic band was observed and that might have resulted in lumen stenosis at day 28 as also reported by Reinertson (1976). In the present study, stenosis at the suture line was more expressed in all anastomotic techniques as compared to previous studies by Singh et al. (1979*b*, 1980, 1985) on the small bowel of ruminants. This might be due to differences in the histology of colon, chronic inflammatory response, purse-string effect of the continuous suture pattern and, of course skill of the surgeon which, in turn, need detailed confirmations.

To sum up, the single-layer inversion technique of suturing was found to be superior to other techniques for colon anastomosis.

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CHANGES IN BLOOD PLASMA BIOCHEMISTRY OF CHICKEN EMBRYOS EXPOSED TO VARIOUS PESTICIDE FORMULATIONS

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Wofatox 50 EC (methylparathion 50%), Nevifosz 50 EC (phosmethylan 50%), Kolfugo 25 FW (carbendazim 25%) and Dikamin D (2,4–D 40%) pesticide formulations were used as test material. The incubated chicken eggs were directly exposed to the applied pesticides with injection into the air cell. Blood samples were obtained and some plasma parameters including packed cell volume (PCV), total protein, glucose, cholesterol, aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and plasma pseudocholinesterase (PChE) activities were evaluated. Although the mortality rate obtained for the treated and control groups did not differ, there were significant changes in plasma biochemistry in relation to pesticide treatment.

The present paper attempts to help those undertaking embryological and teratological studies on avian embryos exposed to pesticides including studies on changes occurring in certain plasma parameters.

Keywords: Blood plasma parameters, chicken embryology, pesticides.

The exposure of hatching, nestling birds to different pesticide formulations causing losses in their population is a continuing problem. Recently there has been an increase in experimental data on biochemical and haematological effects exerted by pesticide on birds (Henderson and Kitos, 1982; Grue and Shipley, 1984; Hoffman et al., 1984; Hoffman et al., 1987; Smith et al., 1986; Rattner et al., 1987). A wide variety of bird species is available for these environmental toxicology studies, e.g. mallard, chicken, pheasant, quail, kestrel and heron. The basic aim of most of these studies has been to evaluate possible hazards constituted by chemical pest control to birds and to get more detailed information on its biochemical and physiological consequences.

This comparative study using developing chicken embryos may shed some light on changes occurring in plasma parameters after direct exposure of chicken embryos to the pesticides applied.

Materials and methods

Fertile chicken (Shaver Starcross 288) eggs from the hatchery of the Agricultural Combinate of Bóly were used. As test material the following pesticides were applied: Wofatox 50 EC (50% methylparathion; VEB Chemie Kombinat, Bitterfeld), Nevifosz 50 EC (50% phosmethylan; NEVIKI, Veszprém), Kolfugo 25 FW (25% carbendazim, Chinoin Pharmaceutical and Chemical Works, Budapest), Dikamin D (40% 2,4-D, Nitrokémia Industries, Balatonfűzfő).

Candled fertile eggs were assigned to one control and three treated groups per test material (Table I) and incubated in a Ragus type incubator under optimum environmental conditions (Kiss, 1977). The pesticides were emulsified in water and diluted to three different dose levels of which the intermediate levels corresponded to concentrations used in the practice of plant protection. Pesticide emulsions were administered by a single injection into the air cell in a volume of 0.1 ml/egg on the 15th day of incubation. Control eggs received the same volume of distilled water.

The applied injection route was not hazardous for the developing embryo and enabled precise dosing. Blood samples were obtained from chicken embryos on the 19th day of incubation. For this purpose eggs were candled to determine the position of the chorioallantoic vessels and a small opening was made. An exposed chorioallantoic vein was nicked and a heparanized capillary tube

Table I

Teratological study of Wofatox 50 EC, Nevifosz 50 EC, Kolfugo 25 FW and Dikamin D in chicken embryos

	Dose, µl/kg egg	Treatment (day of incubation)	Evaluation (day of incubation)	Number of fertile eggs evaluated	Egg weight, g ($\bar{x}\pm SD$)	Fetal weight, g ($\bar{x}\pm SD$)	Number of deformed fetuses
Control	0.00	15	19	20	53.55±3.2	23.78±1.80	0
Wofatox 50 EC	0.55	15	19	15	54.80±2.9	23.28±3.40	0
(methylparathion	5.50	15	19	17	54.20±2.9	24.48±2.60	3
50%)	55.00	15	19	18	55.20±3.7	25.48±2.52	5
Nevifosz 50 EC	0.55	15	19	17	58.70±3.1	22.64±1.94	0
(phosmethylan	5.50	15	19	18	55.10±2.7	24.00±1.90	2
50%)	55.00	15	19	14	53.50±2.6	22.50±1.84	4
Kolfugo 25 FW	0.55	15	19	20	55.10±1.3	24.47±3.20	0
(carbendazim	5.50	15	19	20	54.50±1.3	21.80±2.20	0
25%)	55.00	15	19	20	53.00±1.5	24.00±3.40	2
Dikamin D	1.80	15	19	15	52.66±1.7	25.90±1.60	0
(2,4-D	18.00	15	19	12	51.70±3.2	22.53±4.10	1
40%)	180.00	15	19	14	51.83±3.5	23.80±3.10	2

was filled with blood (Hoffman and Ramm, 1972). After centrifugation and separation of blood plasma, glucose concentration was measured in a glucose analysis system (Zender, 1963). The plasma cholesterol concentration (Watson, 1960), AST (Karmen, 1955), LDH (Babson and Phillips, 1965), and pseudocholinesterase (PChE) activities (Ellman et al., 1961), and plasma total protein (Chromy and Fischer, 1977) levels were determined.

At autopsy all eggs were opened and examined for gross lesions and fetal weights were recorded. Occasional skeletal deformities were identified in preparations stained by the method of Simons and van Horn (1971).

Data obtained from the biochemical analysis were evaluated by Student's *t* test (Finney, 1952).

Results and discussion

The experimental data and the number of altered fetuses are given in Table I. Macroscopically evaluable alterations (i.e. retardation, haemorrhage, crossing beak, cyllosis) were sporadic but showed dose-dependent differences with the pesticide formulations tested. The incidence of malformations was higher for Wofatox 50 EC and Nevifosz 50 EC.

Although fetal weight data did not show significant changes, there were some relationships between plasma parameters. Certain plasma parameters (see Table II) were determined by simple spectrophotometric micromethods. Packed cell volume was similar to that of the control for most treatments, except after exposure to higher doses of the applied organophosphate test materials, when it decreased significantly. Hoffman and Sileo (1984) described the same effect in 18-day-old mallard embryo using EPN (phenyl phosphonothioic acid–0–ethyl–0–/nitrophenyl/ester).

The plasma glucose level was not affected by the treatments in a dose-dependent manner. The 55 μl/kg egg dose of Wofatox 50 EC significantly decreased the plasma glucose level as did the highest dose of Dikamin D.

At the highest doses of Wofatox 50 EC and Kolfugo 25 FW treatments glucose levels significantly increased but remained in the physiological range.

The pesticides applied affected both plasma cholesterol and total protein content but without dose dependency (Table II). The greatest decrease of cholesterol occurred after exposure to Wofatox 50 EC and Dikamin D.

Dikamin D caused a significant decrease in plasma total protein content, while the other test materials resulted in a physiological increase.

AST and LDH activities responded to all doses and the relationships were clearly dose dependent. As described by Hoffman et al. (1987), such an increase of these enzyme activities in birds exposed to pesticides can easily be manifested in higher plasma protein levels. In this study this relationship was absent for the 2,4-D formulation where plasma protein level decreased significantly, as compared to the control, after every dose.

Table II

Blood plasma parameters ($\bar{x} \pm SD$) in chicken embryos exposed to various pesticide formulations (Wofatox 50 EC, Nevifosz 50 EC, Kolfugo 25 FW, Dikamin D)

Pesticides applied	Dose, µl/kg egg	Packed cell volume %	Glucose, mmol/l	AST, U/l	Cholesterol, mmol/l	Total protein, g/l	LDH, U/l	PChE* in % of control
Control	0.00	28.7±1.1	11.0±0.7	24.6±1.8	12.8±1.4	22.3±1.7	450±40	100±15
	0.55	26.8±1.4	10.3±0.6	°30.5±2.1	°6.7±0.7	18.2±2.4	°520±35	87±22
Wofatox 50 EC	5.50	°24.5±1.1	12.5±0.5	°38.4±1.8	°5.9±0.5	21.3±2.0	°580±52	°69±26
	55.00	°25.1±2.3	° 9.1±0.8	°37.4±1.6	°6.5±0.6	°28.7±2.2	°670±45	°62±20
	0.55	25.6±1.2	11.2±0.7	°30.2±1.6	°6.8±0.8	°29.2±2.5	°560±42	84±16
Nevifosz 50 EC	5.50	°22.5±4.0	11.4±0.6	26.8±1.2	11.4±0.4	°24.8±2.3	°690±48	°60±18
	55.00	°22.6±3.8	°12.8±0.5	°30.4±1.4	12.6±0.5	21.6±2.7	°816±74	°48±21
	0.55	26.4±1.6	11.3±0.7	°28.2±2.3	° 7.3±0.7	°26.2±2.5	°580±32	—
Kolfugo 25 FW	5.50	25.8±2.7	11.2±0.7	°36.2±3.4	11.4±2.3	°24.8±2.2	°703±80	—
	55.00	28.1±1.8	°12.4±1.4	°40.0±2.4	12.2±0.2	24.6±2.6	°714±57	—
	1.80	28.4	12.5±1.0	°37.0±3.1	11.7±1.6	°16.0±1.5	°480±30	—
Dikamin D	18.00	27.2	12.5±0.9	°47.0±3.2	° 9.4±1.2	°18.0±2.4	°500±38	—
	180.00	26.1	° 6.8±1.7	°44.6±3.8	° 7.9±1.4	°16.0±1.4	°570±40	—

* = Plasma cholinesterase control value was 624 U/l; ° = significantly different fron control by Student's t test ($P < 0.01$)

The inhibition of plasma PChE activity after exposure to the organophosphate insecticides tested was determined. The decrease in plasma PChE activity was found to be more pronounced after Nevifosz 50 EC treatments. At unchanged plasma PChE levels with low response to an anti-ChE exposure, Hill and Fleming (1982) suggested the detection of plasma butyrylcholinesterase (BChE) because it is usually inhibited more rapidly and in a larger degree than plasma or brain AChE.

In conclusion, direct injection of the applied pesticides into the air chamber of embryonating hen's eggs caused some malformations mostly at higher doses but without constituting a life hazard in this species. The changes found in plasma biochemistry after direct pesticide exposure indicate only a low degree of intoxication. Doses corresponding to those recommended for use in the practice of plant protein proved to be harmless and untoxic. This, however, calls attention to choosing the right dose which is a basic point in integrated pest control.

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BOOK REVIEWS

Hans PLONAIT and Klaus BICKHARDT: *Lehrbuch der Schweinekrankheiten.* Verlag Paul Parey Berlin und Hamburg, 1988. pp. 400, with 192 photographs, 43 colour photographs and 57 tables. Price: DM 98.— ISBN 3-489-57816-3. In German.

Good text-books are those which are intended for a known category of readers. This aspect should consistently be kept in view by authors when writing text-books. In their foreword to this manual the authors unambiguously state that their objective was to describe the clinical picture, to make case histories more comprehensible, and to specify the practical measures to be taken. They have adhered to this clearly specified objective throughout and have subordinated the structure and contents of the book to this aim. As a result, they have written a unique text-book the main advantage of which is its perspicuity and good arrangement (which is a great help in preparing for an exam) and that it shows the way to veterinary practitioners on the basis of the clinical symptoms observed. Instead of listing the recent results of scientific research which are often incomplete yet, with a good sense of criticism the authors systematize the most important knowledge and specify treatment possibilities. Only authors having a wide intellectual horizon, abundant clinical experience and a gift of teaching are capable of such a synthesis. Detailed description of the differential diagnosis of various syndromes is the best proof of their proficiency in their special field. In the reviewer's opinion this detailed treatment of differential diagnosis is the greatest value of the text-book, since in our age characterized by "over-specialization" very few authors shoulder the task of dealing with differential diagnosis in such detail, thus making things difficult for veterinary practitioners.

The text-book is divided into 19 main chapters. The first four chapters deal with the veterinarian's tasks in swine herds, perhaps a little too briefly with management technology and environment. Methods of immobilisation and anaesthetic possibilities in pigs are outlined. Subsequently the clinical interventions and individual treatment of pigs are described, and finally possibilities for mass treatments are outlined in excellent arrangement and practical tables (e.g. dosage of drugs and chemotherapeutics most frequently used against endoparasites and bacteria, related to 1 kg body mass, 1 kg feed, or 1 l drinking water).

Chapters 5 to 18 discuss separate clinical disease entities. These chapters begin with a well-written general introduction which briefly summarizes the pathogenesis, pathophysiology, physiology and diagnostics of the given disease entity. This is followed by description of the aetiology, clinical symptoms, pathologic anatomy and, at praiseworthily great length, aspects of differential diagnosis. Finally therapy and prevention are dealt with.

Division of the book into chapters on the basis of the clinical picture clearly serves the above-mentioned purpose of briefing veterinary practitioners on diseases that may be accompanied by the clinical symptoms and pathological lesions described. Thus the text-book is a valuable aid in establishing the correct diagnosis. Once the diagnosis has been established, the measures to be taken can be decided on.

This arrangement is considered justifiable even if grouping diseases by clinical symptoms cannot be free from the authors' "subjective" approach. In the case of many (mainly infectious) diseases the "main symptom" is not unambiguous, and thus diseases with "mixed symptomatology" are assigned to a chapter rather arbitrarily. This problem may, however, be surmounted by the Contents and the detailed Subject Index. These facilitate reader orientation and every disease can be found easily in the text.

Another debatable point is the length at which a given disease is being treated in the text-book. This is again determined by the authors' attitude. As a specialist of infectious diseases, e.g. I would have liked to read more about several infectious diseases. Of course, one is necessarily influenced by the "order of rank of diseases" characteristic of one's own country. Thus, e.g. the (luckily less and less frequent) diseases caused by *S. typhisuis* and *S. choleraesuis* are judged entirely differently both from the public health and the veterinary point of view. This is not clear from the present text-book. Another point is that veterinary practitioners working for large-scale pig farms would certainly like to read more about enzootic diseases (mycoplasmal pneumonia, swine dysentery, atrophic rhinitis, leptospirosis, Aujeszky's disease). From this point of view Chapter 5, which deals with the reproduction and obstetrics of swine, is especially well written.

On the other hand, due to its brevity, Chapter 19 which deals with the control of disease outbreaks, eradication of infectious diseases, and immunization programmes in swine herds, is rather confined to generalities. The authors aim at systematizing the available knowledge: for details, the reader is directed to the reference list added to each chapter. It is understandable that in a text-book written in German predominantly German authors are cited, since these are the most accessible for the veterinary practitioners. However, in the reference lists there is an abundance of English papers as well. On the other hand, I was sorry to find that authors from Hungary, the homeland of Hutÿra, Marek, Manninger, Mócsy and Aujeszky, are rarely cited, though Hungarian authors have published numerous internationally acknowledged papers (also in German and English) on many pig diseases. Subjectivism is felt also in choosing the publications cited, but, of course, the authors have the right to do so.

With the present volume the Reader receives a well written text-book which will be a valuable aid to the everyday work of veterinary practitioners. The practical and clear arrangement of available knowledge serves this purpose. The 192 photographs, including 43 colour photographs, are good and useful as well as are the 57 tables.

A somewhat more contrasty printing and use of italics would have rendered reader orientation even easier. This valuable and useful text-book would have deserved hard cover.

This text-book can be recommended for veterinarians working in swine practice and for all those who want to systematize their knowledge on swine diseases.

János MÉSZÁROS

Terry W. CAMPBELL: *Avian Hematology and Cytology*. Iowa State University Press, Ames, Iowa, 1988. 100 pp., hardcover, with 226 colour photographs. $ 34.95, ISBN 0-8138-0064-1.

Books on avian haematology and cytology have so far been very much missed by veterinary clinicians and diagnosticians. *Avian Hematology and Cytology* provides a reference for cell identification and the interpretation of avian cellular responses. Cytodiagnosis has long been used in human and domestic mammalian medicine. The use of cytodiagnostic methods for the clinical and pathomorphological study of avian diseases is gaining ground rapidly and such methods may provide essential information for the diagnosis of certain diseases. Thus, this book serves as a valuable reference for specialists and is a useful aid for practising veterinarians and veterinary students.

Chapter 1 describes methods of blood collection from birds and preparation of blood smears, and provides information concerning avian hemic cytology, normal blood count, and abnormal cells in the avian haemogram. *Chapter 2* deals with sample collection from the bone marrow and cytological evaluation of normal avian bone marrow including erythropoiesis, granulopoiesis, monocytopoiesis and thrombopoiesis, whereas *Chapter 3* is devoted to the identification of common blood parasites.

Chapter 4 offers a review of basic veterinary cytotechnology and evaluation of the cytological sample. Basic cytological responses such as inflammation, tissue hyperplasia, and malignant neoplasia are outlined.

Chapters 5 to 10 give a detailed description of the normal and abnormal cytology of the abdominal fluid, oral cavity, oesophagus, ingluvies, cloaca, upper and lower respiratory tract, skin, conjunctiva, cornea, synovial fluid, liver, spleen, kidney, and lymphoid tissue, and of the indications and techniques of sampling. Among other things, the cytological characteristics of abdominal transudate, nonseptic exudate, septic exudate, malignant exudate, haemorrhagic effusion, and abdominal fluid obtained from urate peritonitis (leakage of the urinary tract into the peritoneal cavity) are described. Of diseases of the upper alimentary tract, the cytology and cytodiagnosis of inflammatory lesions caused by traumatic injury, chemical irritation, and infectious agents including different bacteria, fungi and parasites are dealt with, and the

cytodiagnosis of hypovitaminosis A is outlined. Of diseases of the cloaca, ulcerative inflammation, papillomatosis, and certain bacterial, fungal and parasitic infections are described. From the chapters devoted to cytology of the respiratory tract, the cytological characteristics of chlamydiosis, mycoplasmosis, aspergillosis, and cryptococcosis deserve special attention, while in the chapter dealing with cytology of the skin and subcutis the description of pox lesions, certain mycoses and parasitoses as well as neoplastic lesions are outstanding. *Chapter 11* (Cytology of the Conjunctiva and Cornea) describes, among other things, mycoplasmal and chlamydial lesions, whereas *Chapter 12* (Cytology of Synovial Fluid) offers a detailed description of nonseptic and septic arthritis and articular gout. The chapter devoted to normal and abnormal cytology of the liver, spleen, kidney, and lymphoid tissue describes the cytodiagnosis of tuberculosis, chlamydiosis, and certain blood parasites *(Hemoproteus, Leukocytozoon, Plasmodium,* and *Atoxoplasma).*

The final chapter *(Chapter 14)* provides examples of common artifacts that occur on cellular samples and can mimic abnormal cells or infectious agents. The *Appendix* contains a description of routine haematology and cytology stains and staining procedures.

The book also contains a bibliography, a five-page Subject Index, and an alphabetical Glossary.

The material in this book is mostly based on observations made on pet and exotic birds (the Author is currently an assistant professor at the College of Veterinary Medicine, Kansas State University, where he is responsible for the medical and surgical care of exotic animals, especially birds). Since, however, there are many similarities between avian species, the methods and information described make this book a fundamental book of reference in the clinical and pathological cytodiagnosis of poultry diseases, too. The excellent typographical make-up and the colour photographs should be emphasized as the greatest value of this volume.

R. GLÁVITS

Irmgard GYLSTORFF and Fritz GRIMM: *Vogelkrankheiten.* Eugen Ulmer Verlag, Stuttgart, 1987. 609 pp, in German.

Despite the steadily deteriorating environmental circumstances, still some 9,000 bird species exist and make our world more beautiful and variegated. The majority of these species live in the open, in a constant ecological environment or seasonally migrating to very large distances. Many bird species are housed in zoos and an increasing number are kept as pet birds.

The knowledge available on the diseases of this vast "body of patients" has been synthesized by the authors who have also added their own abundant experience. They have fulfilled a very important mission. Although they intended their book for veterinary practitioners and for beginners in this profession, it would be useful to a much broader public.

The authors have solved the taxonomic and nomenclature-related problems, mentioned in the Foreword, very successfully. Their taxonomy, based on the work of *Wolters* (1982), is demonstrated at the beginning of the book in a clearly intelligible and well-arranged, 4-page table indicating the orders, suborders and families in Latin and German. They try to eliminate nomenclature problems by giving, in parentheses, the Latin name of the different bird species mentioned in the German text.

The book is divided into 12 main chapters. Each chapter is subdivided to sections numbered according to the decimal system.

The *general part* summarizes essential anatomical knowledge. The anatomy of the different organ systems is immediately followed by description of their functions. This part of "functional anatomy" demonstrates the interdependence of morphological structure and function. Many clinical and physiological indices of the different bird species are demonstrated in tables which provide very useful information. This chapter also deals with environment, the physiological basis of nutrition, and the nutritional requirements. In my opinion, this important topic would have deserved more detailed treatment in a book which attempts to consider the practical angles.

The authors should be congratulated on a highly valuable part of this chapter, i.e. that describing the methods of clinical examinations and the possible ways of treatment. The indications, doses, possible incompatibilities and side effects of drugs are presented in well-arranged tables.

The chapter dealing with *infectious and invasive diseases* is subdivided into sections devoted to viral, bacterial, fungal and parasitic disceases. As it is stated in the Foreword, the authors confine themselves to describing only the most necessary medical (pathological) knowledge, almost like in a compendium. Readers interested in more detailed descriptions are

referred to any one of the fortunately large number of books available on infectious and parasitic diseases. As an expert specializing in infectious diseases, the reviewer emphasizes two points as the greatest values of these chapters: one is that the authors list the bird species susceptible to the different pathogens. This provides more abundant information than that to be found in the special manuals. This information is very useful from both the epizootiological and the diagnostic point of view. Another valuable part of the chapter is the section where the authors describe diseases occurring in certain bird species only and inform the Reader whether a given disease has been reported from a given bird species. The authors deserve credit for having collected and systematized these important data.

Some statements of this chapter surprised me: e.g. the statement that pigeons do not develop immunity to Newcastle disease if they are immunized with strain La Sota or B_1 administered as a spray or in the drinking water; therefore, in the authors' opinion it is expedient to administer these strains subcutaneously. In Hungary we have more favourable experience with the La Sota strain given in the drinking water, followed by an inactivated oil-adjuvant vaccine than with the inactivated vaccine alone. In our opinion, mosquitoes play an important role in transmitting pigeon pox infection. Young susceptible pigeons become affected by pigeon pox more frequently in places and in seasons of mosquito invasion than elsewhere and at other times. Instead of two attenuated vaccines given 19 weeks apart against duck plague, we have gained very favourable experience with Hungarian attenuated vaccines given twice, at a 2-week interval, before the laying season. More and more authors consider listeriosis a sapronosis rather than a zooanthroponosis. This is justified by the fact that from humans in most cases phage-types of *Listeria monocytogenes* not occurring in animals are isolated.

The diagnosis of infectious diseases is facilitated by carefully compiled tables which may serve as a useful starting point, however, with the limitations such a "schematization" inevitably has. Tables summarizing information on parasitic diseases are extremely useful.

In the chapter dealing with *metabolic disorders and deficiency syndromes* also the successfully constructed tables should be emphasized. Although these "save" much text, I feel that the topic of this chapter would have deserved more detailed treatment. In the metabolic disorders and symptoms of deficiency syndromes there probably are greater differences between bird species than it is suggested by the generalizing statements. Still, the description of clinical symptoms and pathological lesions of organs is remarkable.

The subchapters dealing with *poisoning* and *toxicosis* are excellent (e.g. lead and organophosphate poisoning) and the, unfortunately more and more timely, information on pesticides and herbicides is also very useful. A mistake, however, should be pointed out. It has unanimously been established by many authors in the international and Hungarian literature that *Gallus domesticus* is completely resistant to F-2 toxin; consequently, this toxin cannot cause a mortality rate amounting to more than 40% in chickens. The described symptoms and production decrease can, however, be induced by *any other* mycotoxin.

The chapter dealing with the *diseases of organs* provides abundant and very useful information. This is one of the best chapters and gives much help in the diagnosis of the various syndromes.

Managemental disorders (neuroses) are dealt with very briefly: on two pages only. Bird owners and breeders would certainly have appreciated if these problems had been discussed in more detail.

The same holds for diseases of the *endocrine system* which are also dealt with rather laconically (on 2 pages).

Locomotor diseases are discussed in a well arranged, concise chapter. Further orientation is, however, rendered difficult because only one reference is given.

The chapter on *neoplasms* is complemented by a very useful table and abundant reference list.

Bird surgery is undoubtedly the most beautiful and serviceable chapter of the book. Excellent X-ray pictures acquaint the reader with diagnostics and surgical solutions of the different cases.

The chapter entitled *Diseases of the incubation period* synthesizes the problems of that period and provides the reader with useful particulars on the artifical rearing of young that have to leave the nest.

The comprehensive chapter on *legal regulation associated with birds* gives gap-filling information. Data on environmental protection are of outstanding significance. The compilation on bird species with different levels of protection offers well-arranged data.

Orientation is facilitated by an extensive *Subject index.*

The typographical make-up of the book is excellent. It has 35 beautiful coloured illustrations on separate plates and 61 black-and-white pictures. The 55 tables are the greatest asset

of the book, and the great work done by the authors in compiling and systematizing the data shown in the tables was worth the trouble: they can count on the gratitude of those interested in this subject.

The whole book reflects the authors' great expertise and firm determination to create a uniform and concise work intended for practitioners. Perhaps the reference list given at the end of the chapters does not always reflect this endeavour. The reviewer is sorry to see that, of the large number of English- and German-language publications of Hungarian authors on the topic only one is listed in the references.

The book is not only useful but indispensable for specialists dealing with birds. The authors deserve many thanks and gratitude for this well-written work.

János MÉSZÁROS

Charles M. SCANLAN: *Introduction to Veterinary Bacteriology*. Iowa State University Press, Ames, Iowa, 1st edition, 1988. pp. 457, in English.

The book comprises basic principles of veterinary bacteriology and of the most important bacterial diseases of cattle, sheep, swine, horse, dog and cat. The text is divided into seven sections. (I) *General bacteriology* deals with the taxonomy of bacteria and with the structure and functions of the bacterial cell. (II) *Bacterial pathogenesis* deals with the virulence factors of bacteria, the normal bacterial flora of the skin, mucous membranes etc., with the processes taking place during bacterial infections in the animal body and with the role of the immune system of animals in the prevention or elimination of an infection. (III) The section *Extracellular aad facultative intracellular bacteria* gives detailed account on all bacteria which might cause disease in domestic animals from streptococci to mycoplasma and ureaplasma. (IV) The section *Obligate intracellular and cell-associated bacteria* contains *Chlamydia*, *Rickettsia* and *Anaplasma* species. Section V describes bacterial diseases of cattle, sheep, swine, horse, dog and cat. Aetiology, transmission, clinical features, pathogenesis, pathology, diagnosis of, and immunisation against, each disease are described in a short and concise form. The text of this chapter describes only the very basic facts in respect of each disease and therefore the book can be useful for students and veterinary practitioners but it gives only limited information for teachers or for those who are already familiar with the different diseases. Section VI, entitled *Determinative bacteriology*, gives details of bacterial diagnostic procedures including morphology, staining, culture, growth and biochemical characteristics, aerobic cultivation systems, antimicrobial susceptibility testing methods, etc. Section VII, entitled *Zoonotic bacterial diseases*, gives only a list of zoonoses on two pages. An extensive glossary, as well as a list of selected references is added.

The book in an outline format is a useful one, and fulfils the goals indicated by the author in the preface. It might be used with advantage by veterinary students learning bacteriology. It can serve as a reference basis for the infectious disease and medicine courses, and it gives a concise update and review of veterinary bacteriology for the veterinary practitioners. It should, however, be mentioned that the chapters dealing with the diseases are oriented mainly to users on the American continent. The taxonomy used e.g. for grouping zoonoses is in disagreement with propositions made by the Expert Committee on Zoonoses of the WHO. Therefore, its use as a textbook for undergraduates will have to be restricted to the Americas.

János VARGA

W. BISPING and G. AMTSBERG: *Colour Atlas for the Diagnosis of Bacterial Pathogens in Animals*. Paul Parey Scientific Publishers, Berlin und Hamburg, 1988. 339 pages, 223 figures (180 in colours), 96 tables.

On two columns of the pages the text of the book is presented parallel in English and German. It contains the techniques and methods for the isolation of animal pathogenic bacteria from excretions, organ, tissue and other samples as well as the most important properties of the agents upon which their differentiation might be based in order to reach a reliable bacteriological diagnosis. As outlined in the preface of the book, the authors aimed at a reasonable compromise between the practitioner's requirement for a rapid, economical and clinically useful diagnosis on the one hand, and the bacteriologist's aim at reaching a precise and definitive identification of the isolated agent on the other.

The book describes the bacteria, including chlamydiae, rickettsiae and mycoplasmas, according to the presently accepted systematics. Within one chapter the occurrence and

veterinary significance, the morphology, culture, biochemical properties and, where applicable, antigenic structure and animal experiments to identify the pathogen and to attain a practically useful aetiological diagnosis are described.

The figures of the book are of excellent quality. About one third of them are microphotographs of the agents, the rest are shots of the colonies of the pathogens on various culture media, or of characteristic reactions. The tables contain the most important morphological, cultural, biochemical and other characteristics of the actual agent, compared with data on similar species or those to be considered from the point of view of differential diagnosis. Originality and usefulness from the veterinary medical aspect of information condensed in these tables should especially be mentioned.

A bibliography of the most useful publications on the subject is given at the end of each chapter.

The appendix contains, in addition to a glossary of abbreviations used in the text, the tabulated taxonomy and nomenclature of bacteria of veterinary importance.

The book is an excellent guide for all those engaged in the bacteriological diagnosis of infectious diseases. In addition to veterinarians and physicians working in diagnostic laboratories, the book will be useful for laboratory personnel enganged in bacteriological work on foodstuffs, feed, water and soil as well as in microbiological industry processes.

Bilinguality of the book offers a unique opportunity to familiarize with special technical terms of these two most frequently used languages, mainly to members of the smaller European ethnic groups.

János VARGA

Oxygen Free Radicals and the Tissue Injury. Edited by B. MATKOVICS, D. BODA and H. KALÁSZ. Akadémiai Kiadó, Budapest, Hungary. First edition, 1987. pp. 412, in English.

For two decades, research into the reactions of free radicals has been a rapidly developing field of biology. This is proved by biannual international conferences on the topic after which the papers are published in proceedings.

Hungarian researchers (chemists, biochemists, biologists, physicians, etc.) recognized the significant role played by the reactions of oxygen free radicals in the tissue injury, and extensive studies of these reactions are currently under way. The results are publicized at biannual, so-called Free Radical Conferences held in Hungary where primarily the adverse effects of oxygen free radicals are analysed.

The present volume contains the proceedings of papers read at the 2nd Free Radical Conference held in Szeged in 1986. From the title one could assume that this book is a monograph. However, from the Introduction it becomes clear that the volume contains the proceedings of the 2nd Free Radical Conference. It would have been good if this fact had been indicated at least as a subtitle.

The proceedings of the 44 papers read at the Conference are given in alphabetical order of the authors' names. The papers are not grouped by subject. This gap is filled by a detailed "Subject Index" at the end of the book which facilitates reader orientation.

The decisive majority of the authors is Hungarian. They describe *original research* results in abstracts running to some pages. The book contains, however, some *reviews* as well.

The extensive relationship existing between free radical reactions and the tissue injury is reflected in the diversity of the papers. Several abstracts deal with the *general* importance of free radicals in medical practice. Most abstracts discuss the role of free radicals in connection with the pathogenesis of hepatopathies (including alcohol-induced liver injury), muscle injuries and rheumatic changes. New data are reported also on lipid-peroxidative processes influencing the function of red blood cells and different white blood cell types. The research results are illustrated by clearly arranged figures and tables. Reader orientation is facilitated by a detailed reference list after each subject.

None of the papers is directly related to veterinary practice, suggesting that the significance of free radical reactions was first recognized in connection with tissue injuries occurring in the human organism also in Hungary. This is even more thought-provoking as in veterinary medicine syndromes (e.g. myodegeneration caused by vitamin E or selenium deficiency) positively associated with enhanced lipid-peroxidative processes have been known for several decades (!). Therefore, the book can be recommended not only for physicians and chemists but also for veterinarians dealing with free radical reactions. This book provides *general knowledge* on the significance of free radical reactions, but also serves as *concrete help* for those engaged in fundamental or applied research.

Tibor GAÁL

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CONTENTS

Acta Veterinaria Hungarica 37 (3), pp. 191–196 (1989)

A NEW STAINING AND EVALUATING PROCEDURE FOR PROTEIN GEL ELECTROPHEROGRAMS BASED ON THE PYROGALLOL RED–MOLYBDATE COMPLEX

A. Csiba and L. Szécsényi-Nagy

Department of Medicine and Clinical Pharmacology, Municipal Hospital Péterfy, H-1441 Budapest, Péterfy S. u. 14, Hungary

(Received October 15, 1988)

A new method is reported for staining and evaluating gel electropherograms of proteins.

With pyrogallol red–molybdate reagent the gel-embedded proteins are transformed into a derivative of blue colour. After destaining, the blue-coloured proteins are well visible against a colourless background and can be quantified by densitometry with high reliability.

The quantity of the coloured protein is directly proportional to the height of peaks in the densitogram. Colour intensity is concentration dependent.

The measurement range of serum albumin was 1 to 50 μg/tube and 10 to 100 μg/slab in polyacrylamide gel disc electrophoresis and agar gel electrophoresis, respectively.

Keywords: Protein, gel electropherogram, staining procedure, pyrogallol red–molybdate complex.

Numerous staining procedures are known for visualization of proteinograms. These are described in detail in manuals e.g. Kerese, 1975; Dévényi and Gergely, 1971.

After the completion of gel electrophoresis the gels are fixed, stained and washed. The most frequently used staining procedures are based on the dye-binding capacity of proteins, i.e. binding of the dye colloids to proteins by electrostatic and van der Waals forces. Examples of this are the amido black and the different coomassie brilliant blue dyes. For visualization of proteins, coomassie brilliant blue dyes are ten times as sensitive as amido black. There exist staining procedures which are based on a colour reaction instead of colloid binding. In these procedures the developer reacts with some functional group of the protein, resulting in a coloured derivative. To this category belong the so-called specific staining procedures developed for demonstrating non-protein substances bound to protein.

The protein staining procedure described here has been developed on the basis of a colorimetric method of Japanese authors (Fujita et al., 1983; Watanabe et al., 1986) who used it for determining the concentration of urinary proteins in different syndromes. Using their pyrogallol red–molybdate reagent, urinary proteins can be determined at 600 nm wavelength. We were the first in the world to use this reagent for visualization of protein gel electropherograms.

Materials and methods

Materials

Disodium molybdate (VI) $\times 2\ H_2O$, succinic acid, sodium oxalate, sodium benzoate, trichloroacetic acid, methanol, 96% acetic acid and 37% hydrochloric acid (all of analytical grade) were obtained from Reanal Fine Chemicals Co. (Budapest, Hungary). Pyrogallol red was purchased from Serva (Heidelberg, FRG) and human serum albumin standard from Sigma (St. Louis, USA). The chemicals necessary for gel preparation were obtained from a kit marketed by Reanal Fine Chemicals Co. (Budapest, Hungary). For preparing the electrophoretic buffer and gel systems double-distilled water was used.

Instruments

Gel electrophoresis was performed with the Model-73 apparatus marketed by Reanal Fine Chemicals Co. (Budapest, Hungary). The electropherograms were scanned with an OE-508 type densitometer (Labor MIM, Budapest, Hungary) and evaluated with the help of the Labsys 80 personal computer program (Labor MIM, Budapest).

Method

Polyacrylamide and/or agar gel electrophoresis was performed according to the practical "Instructions for Use" attached to the instrument by Reanal Fine Chemicals Co. (Budapest, Hungary). In brief, the concentration of the polyacrylamide gel was adjusted to 7% by the method of Davis (1964). A buffer system of pH 8.9 was used. Agar gel electrophoresis was performed in a "micro gel" system (Wunderly, 1957). Agar concentration was adjusted to 1.5% before pouring out the slab. An electrophoretic buffer system of pH 8.2 to 8.4 was used.

Staining procedure

Fixative: Protein fractions in the gels were fixed in 12.5% aqueous trichloroacetic acid.

Staining solutions: Pyrogallol red solution (1.5 mM): Dissolve 60 mg pyrogallol red in 100 ml methanol. Molybdate solution (0.01 M): Dissolve 0.24 g disodium molybdate $\times 2\ H_2O$ in 100 ml distilled water.

Pyrogallol red–molybdate reagent: Dissolve 5.9 g succinic acid, 0.14 g sodium oxalate and 0.5 g sodium benzoate in 900 ml distilled water, then add 40 ml pyrogallol red solution and 4 ml molybdate solution. Adjust the pH of the mixture to 2.5 by adding 0.1 M HCl and make the mixture up to 1 litre with distilled water.

Staining procedure: After the run the gels are immersed in 12.5% trichloroacetic acid of tenfold volume for 30 min. Subsequently they are transferred into pyrogallol red–molybdate reagent. After staining for 5 h the gels are washed with water, then immersed in 7% aqueous acetic acid. With the acetic acid the excess orange-red reagent can easily be removed from the gel. Change the washing fluid only twice (as long as it shows discoloration). After destaining, the protein-free parts of the gel become colourless and transparent. The blue colour of the protein-containing gel parts becomes more intensive in the destaining solution. The gels can be stored in 7% acetic acid for 1 month.

Results and discussion

To determine the sensitivity of the staining procedure, different volumes of standard human serum albumin were applied on the gels prepared as speci-

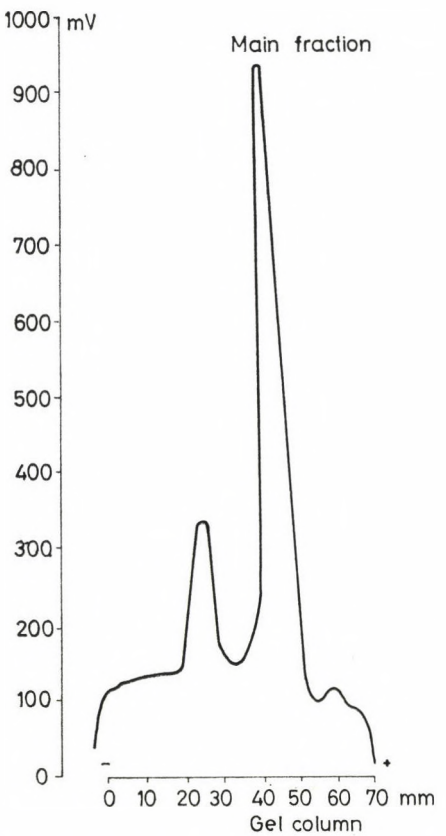

Fig. 1. Disc gel electropherogram of human serum albumin. Protein quantity applied on the gel: 50 μg/tube. Densitometer density adjustment: 1. Applied filter: 630 nm. Measurement limit of the recorder: 1000 mV

fied in the "Instructions" issued by Reanal. After electrophoresis at a current intensity of 5 mA/tube and staining of the proteins, the proteinograms were evaluated by densitometry.

This preliminary experiment showed that in polyacrylamide disc electrophoresis the optimum measurement range is 1 to 50 μg albumin per tube. In that case the density of electropherograms is in linear relationship with the quantity of gel-embedded albumin. The sensitivity of this procedure is superior to that of the conventional amido black and coomassie brilliant blue staining procedures.

Figure 1 shows the polyacrylamide gel disc electropherogram of human serum albumin (50 μg protein/tube). The human serum albumin preparation (Sigma) contains two easily detectable fractions (peaks). The first (fast-moving) component is about five times that of the one following it.

For calibration, the electropherograms of 1, 5, 10, 20, 30, 40 and 50 μg/tube human serum albumin were scanned with a densitometer. Data calculated with a Labsys 80 computer show that the densitometric peak heights expressed in mV are in linear relationship with the protein quantities layered on the separating gel. The calibration relationship is shown in Fig. 2.

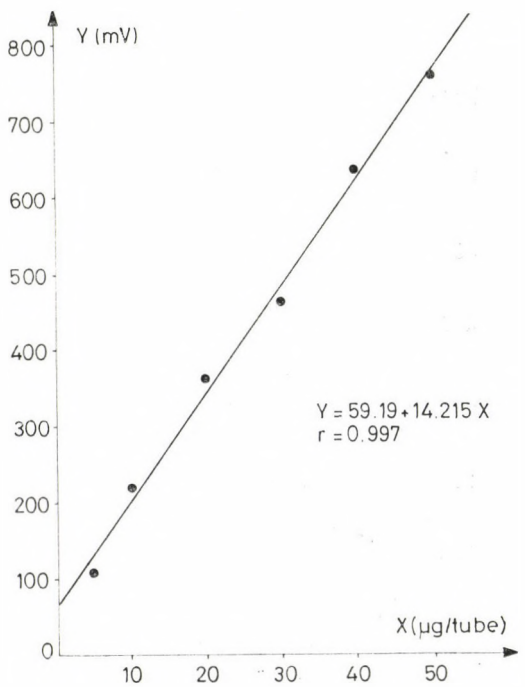

Fig. 2. Calibration with human serum albumin on the basis of its disc gel electropherogram. Densitometer density adjustment: 1. Applied filter: 630 nm. Measurement limit of the recorder: 1000 mV

The measurement points shown in Fig. 2 fit well (with a reliable correlation) the regression line constructed with the help of a Commodore 64 program.

When the calibrating protein quantities were developed with coomassie brilliant blue, the pale "protein bands" could not be evaluated because of the unbleachable background.

Our procedure was also used to develop agar gel electropherograms. Serum samples of 7 μl were applied onto the slabs. After the run the slabs were developed and evaluated by densitometry.

Figure 3 shows the densitogram of an agar gel electropherogram developed on the slab. The densitogram shows that albumin was the predominant serum protein component. With the reagent used, not only albumin but also the other serum protein components can be demonstrated.

The advantages of our procedure developed for staining gel-embedded proteins can be summarized as follows:

(1) The orange-red coloured reagent diffuses into the gel as a solution and forms a blue complex with (instead of adsorbing to) the protein.

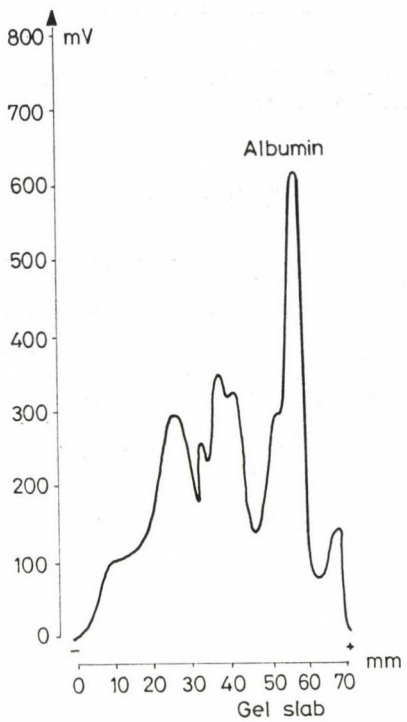

Fig. 3. Agar gel electropherogram of human serum. Serum volume: 7 μl/slab. Densitometer density adjustment: 2. Applied filter: 590 nm. Measurement limit of the recorder: 1000 mV

(2) Proteins are developed with high sensitivity in the entire cross-section of the gel. After the appearance of the blue colour, the excess reagent that has diffused into the gel can easily be removed.

(3) After destaining, the blue-coloured proteins can be seen against a colourless background.

(4) The quantity of coloured proteins is directly proportional to the peak heights of their densitogram, i.e. the colour intensity is concentration dependent.

(5) Protein development is highly sensitive: the measurement range is 1 to 50 μg per tube in disc electrophoresis and 10 to 100 μg per slab in agar gel electrophoresis.

In this work we did not aim at completeness. Instead, we wanted to describe this new gel staining procedure as soon as possible, assuming that, owing to the colourless background and extremely high sensitivity, the procedure would command great interest.

Comparison of this technique with other available protein staining procedures is currently under way, with special respect to the different protein types.

Acknowledgements

The authors wish to thank Dr. Sándor Juhász, Chemical Engineer, for his encouragement in propagating the procedure reported in this paper.

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CLINICAL AND MICROBIOLOGICAL STUDIES OF *DERMATOPHILUS CONGOLENSIS* INFECTION IN CATTLE

(SHORT COMMUNICATION)

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Dermatophilosis (streptotrichosis) is an infectious, cosmopolitan disease of major economic importance as it results in substantial losses of hides, skin, wool, beef and milk (Ainsworth and Austwick, 1973; Lloyd, 1986).

A total of 140 animals comprising 74 Jersey, 57 Jersey × Kankrej (an indigenous breed), 5 Holstein-Friesian and 4 Holstein-Friesian × Kankrej were examined for the presence of *Dermatophilus* infection. They ranged in age from 2 months to 10 years. Specimens were collected from 45 animals and from 6 animal handlers. The samples were directly smeared on duplicate slants and Petri dishes of blood agar, brain-heart infusion (BHI), Sabouraud's dextrose agar with chloramphenicol (0.05 mg/ml) and Sabouraud's dextrose agar with chloramphenicol and actidione (0.5 mg/ml). The cultures were incubated aerobically and anaerobically in a candle jar at 37 °C. Direct microscopy was done in 11 cases by Giemsa and potassium hydroxide (KOH) techniques. In addition, 5 samples each of straw, hay, millet, soil and dung were examined by the dilution method. The isolates were identified by the procedures recommended by Gordon (1964).

Thirty-six out of the 140 animals examined (25.7%) were found to be clinically affected. Twenty-six (72.2%) of the affected cattle showed crusted lesions on the leg, back and rumps, and 7 (19.4%) had generalized lesions involving the head, face, neck, dewlap, brisket, thigh, axilla, abdomen, coronet, leg, tail, scrotum and udder. In 3 lactating cows systemic signs such as anorexia, fever, salivation, reduced milk yield, abortion and retention of placenta were also noticed.

Giemsa-stained smears of exudate and scab of crusts in 11 animals revealed the presence of thin, branched filaments with cocci and chains morphologically resembling *D. congolensis*.

The pathogen was isolated from 8 out of the 51 skin scrapings and crusted materials on blood agar and BHI after 72 h of incubation at 37 °C (Table I). The organism could not be recovered from the Holstein-Friesian cattle, their crossbreds, animal attendants, and environmental materials.

Table I

Isolation of *Dermatophilus congolensis* from cutaneous lesions

Source of specimen	Number examined	Recovery of organism on	
		blood agar	BHI agar
Jersey	29	7/29*	4/29
Jersey crossbred	7	1/7	1/7
Holstein-Friesian	5	0/5	0/5
Holstein-Friesian crossbred	4	0/4	0/4
Animal attendant	6	0/6	0/6
Total:	51	8/51	6/51

* Numerator indicates number of specimens positive for the pathogen and denominator indicates number of specimens examined

In the absence of any parasites, fungi and bacteria, the findings of the present investigation confirmed that *D. congolensis* was the only pathogen incriminated in the aetiology of dermatitis. The disease was confined to the Jersey cattle and their crossbreds, as the Holstein-Friesian cattle and their crossbreds were housed in a separate shed.

Though the clinical signs of the disease were as described earlier by other investigators (Szabuniewicz, 1964; Drager and Vanalsting, 1985), the literature revealed paucity of information on systemic signs such as abortion and retention of placenta. In this outbreak, three Jersey cattle with generalized cutaneous lesions aborted and retained the placenta. As clinical materials were not available from the aborted animals, the aetiological role of *D. congolensis* in abortion cannot be unequivocally proved.

The zoonotic significance of dermatophilosis was reported by Dean et al. (1961). However, in the present investigation *D. congolensis* could not be detected in any of the 6 animal attendants who had close contact with the diseased cattle. No experimental study was undertaken to prove the pathogenicity of the organism in human beings.

Acknowledgements

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EFFECT OF SELENIUM, VITAMIN E AND RIBOFLAVIN SUPPLEMENTATION OF THE FEED ON THE HUMORAL AND CELL-MEDIATED IMMUNE RESPONSES OF GROWING PIGS

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The feed of weaned piglets of Hungarian Large White × Duroc and Dutch Landrace × Duroc genotype was supplemented with 0.5 mg selenium, 50, 100 or 150 mg vitamin E, and 2.5 or 5 mg riboflavin per kg.

Feed supplementation enhanced the cytotoxic reaction and elevated the antibody titres produced against purified horse gamma globulin antigen. However, as compared to the control the differences were not significant. Feed supplementation exerted a beneficial, though varying, influence on the indices of cell-mediated immunity. The proportion of rosette-forming cells and blastogenic transformation induced by specific (horse globulin) and nonspecific (phytohaemagglutinin, PHA) mitogens underwent the most expressed and most significant increase in pigs fed 5 mg selenium, 100 mg vitamin E and 5 mg riboflavin per kg of feed. On the other hand, feed supplementation failed to enhance the responsiveness to intradermal PHA (type IV allergic reaction).

Keywords: Selenium, vitamin E, vitamin B_2, riboflavin, selenium concentration, liver, serum, adrenal cortex function, humoral immune response, cell-mediated immune response, lymphocyte stimulation, rosette formation, intradermal PHA, cytotoxic reaction.

The genetically determined functioning of the immune system and defence mechanisms of farm animals can be influenced by neuroendocrine function, immunosuppressive and immunostimulant substances, and numerous dietary factors. Parallel with the spread of closed pig management systems and acidification of soils, an increasing number of papers have dealt with losses caused by selenium (Se) and vitamin E deficiency (van Vleet, 1980; Oldfield, 1985; etc.). Selenium, vitamin E and riboflavin are known to be essential components of the oxidation–reduction system responsible for eliminating the peroxides produced during intermediary metabolism. Several authors (Tengerdy and Nockels, 1975; Nockels, 1979; Ullrey, 1981; etc.) have suggested that Se and vitamin E administered in excess may have a favourable influence on certain components of the immune system and may be important in the prevention of pig diseases of complex aetiology. Though numerous works have dealt with the effect of Se and vitamin E supplementation of pig feeds, to the best of our knowledge the combined effect of Se, vitamin E and riboflavin

in pigs has not yet been studied. The aim of this study was to establish whether supplementation of grower diets usual in Hungary with different doses of Se, vitamin E and vitamin B_2 would alter the humoral and cell-mediated immune response in growing pigs.

Materials and methods

A total of 48 weaned piglets of Hungarian Large White × Duroc and Dutch Landrace × Duroc breed construction, randomly allotted to six groups, were used. The piglets were reared in one-storey batteries in climatized chambers ensuring optimum microclimate corresponding to their age and body mass. Each group contained both male and female piglets of about the same body mass. The health status of the piglets was monitored for a week. Subsequently, their diet (a piglet diet of 0.13 mg/kg selenium, 6.09 mg/kg vitamin E, and 1.86 mg/kg riboflavin content) was supplemented with a selenium, vitamin E and vitamin B_2 containing concentrate, so that the piglets received the following selenium, vitamin E and vitamin B_2 quantities, in addition to the original Se, vitamin E and riboflavin content of the basal ration:

Group	Se mg/kg	Vitamin E mg/kg	Vitamin B_2 mg/kg
1	0.5	50	2.5
2	0.5	100	2.5
3	0.5	50	5.0
4	0.5	100	5.0
5	0.5	150	5.0
6	control group receiving a diet without supplementation		

The immunological profile tests were started on day 28 of feeding the supplemented diets. On day 7 the two largest and two smallest piglets of each group were killed and the Se content of their liver was determined. The immunological profile test was completed on day 17 after the piglets' first antigen treatment. The experimental diets were fed for 76 to 83 days, then the piglets were killed and the Se content of their liver was determined. Blood samples taken on day 74 of the feeding trial were assayed for serum vitamin E concentration. The time course of the experiment is shown in Fig. 1.

The piglets were weighed individually every week. The control and experimental diets were fed *ad libitum*. Feed consumption was determined as the difference of the feed quantity offered and the leftovers. Specific feed utiliza-

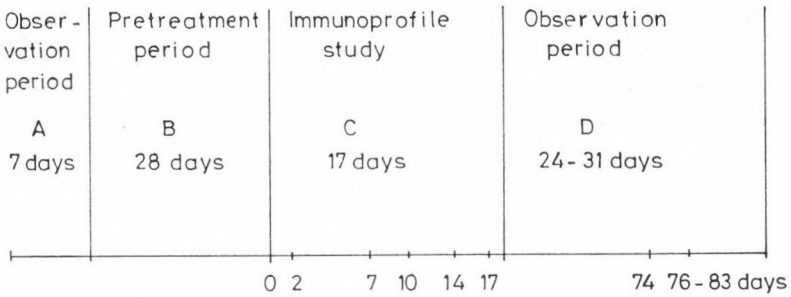


Fig. 1. Design of the experiment. *A*: Observation period (7 days). *B*: Pretreatment period (28 days): experimental diets are fed, the piglets are weighed weekly, and their feed consumption is determined. *C*: Immunoprofile study (17 days). Day 0: immunization with horse gamma globulin. Days, 0, 7, 10, 14 and 17: blood sampling for determining humoral antibodies and for cellular tests. Days 2–4: intradermal PHA test. Day 7: two piglets are killed in each group. Day 10: repeated immunization with horse gamma globulin. *D*: observation period (24–31 days). On day 74 of feeding the experimental diets: blood sampling for assay of serum vitamin E concentration. On days 76–83 of feeding: the piglets are killed and hepatic Se concentration is determined

tion of the piglet groups was calculated from the body mass change and the feed consumed.

The *humoral immune response* was monitored by titrating the antibodies produced against an aluminium hydroxide gel adsorbed, purified horse gamma globulin antigen produced by us. On day 0 and 10 of the immunological profile test 5 ml of the horse gamma globulin antigen was injected intramuscularly. The anti-horse globulin antibodies were titrated by an ELISA developed by the Veterinary Medical Research Institute, Hungarian Academy of Sciences, in blood samples taken on days 7, 10, 14 and 17 after the first antigen treatment.

For the *in vitro cellular tests*, the cells were separated with Ficoll-Paque from blood samples taken into heparinized tubes on day 0, 7, 10, 14 and 17 of the immunoprofile test. Cell density was adjusted to 10^6/ml in Hanks's solution containing 10% fetal calf serum, and 1.8 ml aliquots of the cell suspension were measured into Leighton tubes.

In the *lymphocyte stimulation test* the rate of antigen-specific blastogenesis was studied by adding 0.2 ml of 2% horse gamma globulin, while nonspecific stimulation was determined by adding 200 gamma/0.2 ml phytohaemagglutinin (PHA). The stimulated cell suspensions were incubated at 37 °C for 72 h. In the 56th hour of incubation 10 μCi ^{3}HTdR was added to each sample, then the plates were treated with Ilford Nuclear (CIBA GEIGY Co., Basel) emulsion. The rate of blastogenic transformation was determined by autoradiography on the basis of ^{3}HTdR incorporation (Varga, 1973; Rodák, 1976; Zweimann, 1978).

The *rosette formation test* (RCF) was done by the method of Grewal (1976) and Tilz (1978). Washed sheep red blood cells (RBCs) were pretreated with a 1 : 250,000 dilution of tannic acid, and horse gamma globulin was adsorbed

to their surface. Then the RBCs were repeatedly washed, and 0.1 ml of a 3% RBC suspension was incubated with 1.8 ml of a lymphocyte suspension of 10^6/ml density at 37 °C. Smears were fixed with methanol in the 16th hour, stained with May-Grünwald stain, and used for determining the proportion of rosette-forming cells.

The *cytotoxic reaction* was performed by the method of Kiss and Tuboly (1981) in a xenogenic system. The target cells were sheep RBCs on the surface of which horse gamma globulin had been adsorbed.

In vivo cell-mediated immunity was monitored by intradermal PHA test (Blecha et al., 1983). After measuring the thickness of the skin fold, 0.1 ml saline, containing 250 μg PHA (Difco Laboratories, USA) per ml, was injected into the skin of the inguinal skin fold. Twenty-four and 48 h after the injection the thickness of the skin fold was measured again with a slide-gauge, and the reaction was evaluated on the basis of skin fold thickness.

The Se content of the feed and of liver samples of the killed piglets was determined, after pretreatment with nitric acid and perchloric acid, by fluorometry (Olson, 1973). Serum vitamin E concentration was measured by spectrophotometry, according to the method of Sándor and Csörögi (1989), whereas riboflavin concentration of the feed was determined by the method described by Bokori and Andrásofszky (1985). In blood samples taken between 9 and 10 a.m. on day 0 of the immunoprofile test, cortisol concentration of the peripheral blood was determined once by the RIA procedure used by us for a long time (Rafai and Fodor, 1980).

Results

The mean Se, vitamin E and riboflavin intake of the control group (Group 6) and experimental groups is shown in Table I. The data were calculated from the Se, vitamin E and riboflavin concentration of the control and experimental diets, the daily feed consumption and the mean body mass of the groups.

Figures 2 and 3 show the mean Se concentration of the liver of piglets killed on day 35 after the beginning of feeding and at the end of the trial (on day 76 to 83 of feeding; two piglets each). Comparing the data of Table I and Fig. 2, it can be established that, disregarding Group 3, Se supplementation of the diet increased liver Se concentration by 0.14 to 0.34 mg/kg on the average. The standard deviation (SD) of data shown in Fig. 2 is rather high, partly because these data are the mean of as few as two piglets each. The Se concentrations determined at the end of the experiment are much more uniform, indicating that Se supplementation of the feed was reflected also in the higher Se concentration of the liver.

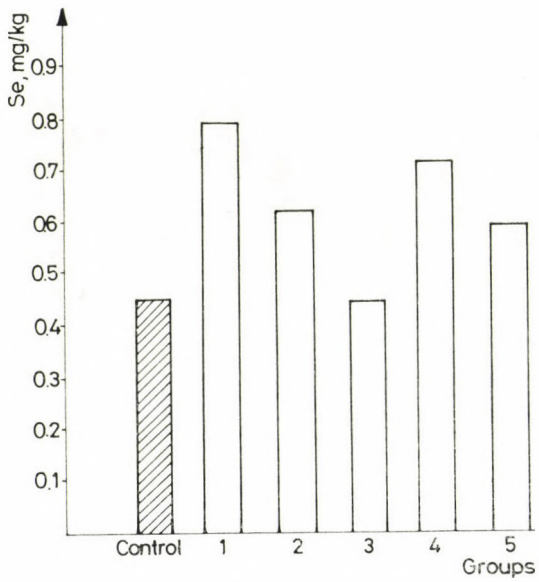
Table I

Mean selenium, vitamin E and riboflavin consumption of the piglet groups during pretreatment and during the immunological profile study (mg/kg body mass)

Control	Selenium	
	Pretreatment period	Immunological profile study
	0.007–0.009	0.004–0.006
1	0.035–0.044	0.035–0.039
2	0.036–0.051	0.022–0.034
3	0.036–0.046	0.023–0.033
4	0.037–0.046	0.023–0.033
5	0.033–0.044	0.020–0.030
Control	Vitamin E	
	Pretreatment period	Immunological profile study
	0.34–0.39	0.19–0.29
1	3.21–3.91	3.09–3.45
2	6.12–8.69	3.76–5.74
3	3.17–4.06	2.06–2.89
4	6.19–7.71	3.89–5.53
5	8.11–10.82	5.05–7.30
Control	Riboflavin	
	Pretreatment period	Immunological profile study
	0.093–0.120	0.09–0.083
1	0.242–0.303	0.241–0.268
2	0.252–0.357	0.155–0.236
3	0.388–0.496	0.252–0.354
4	0.400–0.498	0.252–0.357
5	0.356–0.475	0.221–0.321

A good correlation was found between mean vitamin E concentration of the experimental feeds and that of serum samples taken on day 74 of feeding. The lowest serum vitamin E concentration was obtained for the control group fed the unsupplemented diet. In Groups 1 and 3, groups fed a diet supplemented with 50 mg vitamin E per kg, serum vitamin E concentration was higher. Serum vitamin E concentration was the highest in Groups 2, 4 and 5, groups fed a diet supplemented with 100 (Groups 2 and 4) and 150 (Group 5) mg/kg vitamin E (Fig. 4).

The mean body mass, mean daily body mass gain, and mean daily body mass gain calculated for metabolic body mass of the piglet groups are given in Table II. In the first 28 days of feeding the experimental diets the pigs of Groups 2 and 4 showed significantly lower body mass gain than the controls.

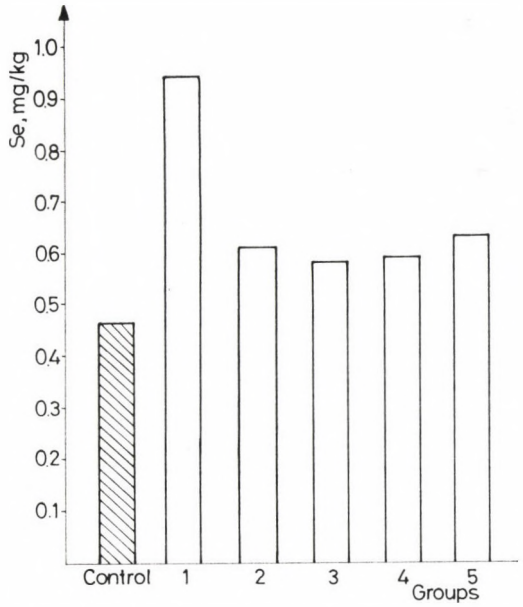


Group	Control	1	2	3	4	5
n	2	2	2	2	2	2
$\bar{x} \pm SD$	0.45 ± 0.03	0.79 ± 0.2	0.62 ± 0.03	0.44 ± 0.04	0.71 ± 0.08	0.59 ± 0.03
$p \leq$		NS	0.05	NS	0.05	0.05

Fig. 2. Hepatic Se concentration (mg/kg) of piglets of the six groups on day 35 of the study. (Due to the small number of piglets within the groups, the data provide little information)

During the immunoprofile study all experimental groups had smaller body mass gain than the control group. Taking the entire experiment (day 1 to 42), in body mass gain only Group 5 was not significantly different from the control (though also this group had smaller mean body mass gain than the control).

As the feed consumption of the weaned piglets was not restricted during the experiment, the smaller body mass gain of the experimental groups may have been due either to Se, vitamin E and riboflavin supplementation of the diets or to body mass differences between the groups at the beginning of the experiment. Covariance analysis revealed that the various experimental groups had significantly differed from one another already on day 1 of feeding, and that differences in body mass gain could be explained by differences in the initial body mass of the groups. This statement is supported by the lack of significant difference between the experimental groups in daily body mass gain calculated for metabolic body mass.



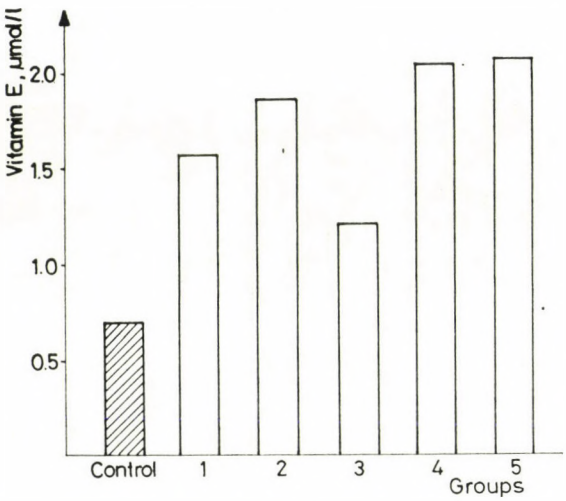
Group	Control	1	2	3	4	5
n	6	4	6	6	5	6
$\bar{x} \pm SD$	0.46 ± 0.03	0.94 ± 0.55	0.61 ± 0.18	0.58 ± 0.09	0.59 ± 0.09	0.63 ± 0.09
$p \leq$		NS	NS	0.02	0.02	0.01

Fig. 3. Hepatic Se concentration (mg/kg) of piglets of the six groups on day 76–83 of the experiment

The cortisol concentration of venous blood from piglets is shown in Fig. 5. As plasma cortisol concentration was between 31.3 and 33.0 ng/ml in the different groups, there were no significant differences in adrenal cortex function between the groups.

Feed supplementation exerted a favourable effect on the anti-horse gamma globulin titres of growing pigs (Table III), though the mean antibody titres of the experimental groups did not differ appreciably from that measured in the control blood samples taken at the same time.

The results of lymphocyte stimulation with horse gamma globulin are shown in Fig. 6, whereas those of nonspecific stimulation with PHA are given in Fig. 7. These figures clearly show that the LST values were more favourable in the experimental groups. The difference from the control was the most favourable and the proportion of rosette forming cells was the highest (Fig. 8) in Group 4. Results of the cytotoxic reaction tended to be higher in the experi-



Group	Control	1	2	3	4	5
n	8	7	8	8	8	8
$\bar{x} \pm SD$	0.7 ± 0.0	1.57 ± 0.54	1.88 ± 0.39	1.20 ± 0.23	2.04 ± 0.65	2.06 ± 0.42
$p \leq$		0.001	0.001	0.001	0.001	0.001

Fig. 4. Serum vitamin E concentrations (μmol/l) on day 74 of the experiment

mental groups than in the control, indicating that supplementation of the feed with Se, vitamin E and riboflavin facilitated the elimination of foreign cells from the organism. Intradermally administered PHA induced type IV hypersensitivity response in all four groups. This was most expressed in the control group and in Group 5 and the least expressed in Groups 1 and 2.

Discussion

After weaning, Se (Mahan et al., 1977) and vitamin E (Mahan and Moxon, 1980) are rapidly eliminated from the piglets' organism. If the feed is not supplemented with Se, Se excretion will exceed Se uptake (Mahan, 1985). As a result, the Se and vitamin E concentration of the tissues will decrease rapidly, reaching the lowest value 6.6 days after weaning on the average (Mahan and Moxon, 1980). In the period around weaning, stress factors and riboflavin deficiency aggravate the effects of Se and vitamin E deficiency (Brady et al., 1979; cited by Peplowski et al., 1981).

Given this knowledge, numerous authors have tried to supplement the diet of weaned piglets with Se and/or vitamin E, and to monitor the effects

Table II

Mean body mass, mean daily body mass gain and mean daily body mass gain calculated for metabolic body mass

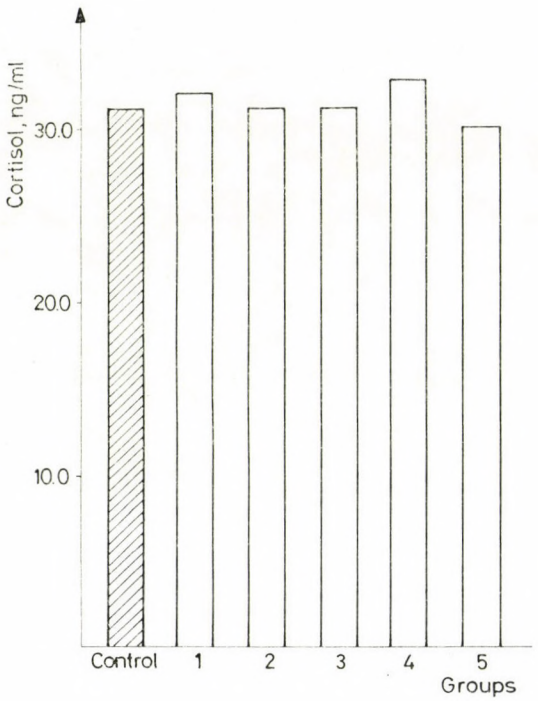
Group	Number of piglets	Mean body mass of the group on day		
		1	28 of the experiment	42
Control	6	13.5 ± 0.6	25.4 ± 1.8	34.3 ± 1.5
1	6	9.6 ± 1.1	20.0 ± 1.6	26.1 ± 1.9
2	6	10.1 ± 0.4	20.3 ± 1.4	28.1 ± 1.8
3	6	11.1 ± 0.5	22.3 ± 1.8	29.6 ± 2.1
4	6	11.3 ± 0.5	22.0 ± 1.5	29.4 ± 1.3
5	6	12.7 ± 0.4	24.7 ± 1.2	32.5 ± 1.0

Group	Number of piglets	Mean daily body mass gain, g/day		
		day 1–28	day 28–42	day 1–42
Control	6	457 ± 36	685 ± 41	520 ± 35
1	6	397 ± 62	476* ± 143	433* ± 51
2	6	391* ± 51	603* ± 65	454* ± 40
3	6	432 ± 42	559* ± 90	462* ± 47
4	6	411* ± 34	569* ± 85	457* ± 33
5	6	462 ± 47	600* ± 61	495 ± 30

Group	Number of piglets	Mean daily body mass calculated for metabolic body mass, g/day body mass kg 0.75		
		day 1–28	day 28–42	day 1–42
Control	6	49.4 ± 3.2	53.7 ± 2.4	48.2 ± 2.4
1	6	52.6 ± 6.8	45.2 ± 12.7	49.8 ± 5.6
2	6	50.7 ± 4.9	55.2 ± 5.2	51.6 ± 7.3
3	6	52.6 ± 3.9	48.5 ± 6.7	52.0 ± 7.9
4	6	51.0 ± 3.9	49.8 ± 6.8	47.5 ± 2.5
5	6	51.3 ± 4.5	49.4 ± 6.6	47.8 ± 2.2
F value		48.06***	17.61***	18.36***
SD 5%		0.61	1.52	1.98
1%		0.82	2.05	2.68
0.1%		1.09	2.73	3.55

* Difference from the control is significant at $P < 0.05$
*** The F value indicates significance of $P < 0.01$

of this supplementation. As the main site of Se storage is the liver (Behne and Höfer-Bosse, 1984), the effect of Se supplementation is often characterized by the changes in hepatic Se concentration. The experiments of Mahan (1985) proved that supplementation of piglet diets with 0.3 to 1.0 mg Se per kg of feed resulted in a linear increase of the piglets' Se retention. This increase was directly proportional to the Se supplementation. According to Meyer et al. (1981), peak blood plasma and hepatic Se concentrations are achieved at a dietary Se concentration of 0.5 to 1.0 mg Se/kg of feed. Increased riboflavin concentration of the feed was found to reduce urinary Se excretion, which exerts a favourable influence on Se retention and increases the glutathione

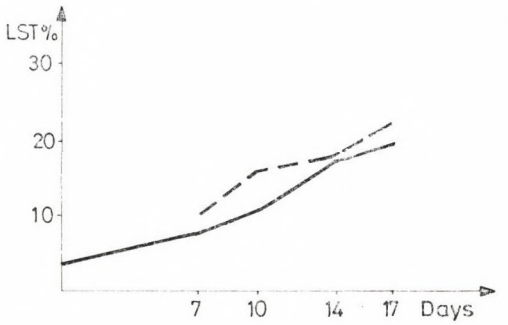


Group	Control	1	2	3	4	5
n	8	6	6	8	6	8
$\bar{x} \pm SD$	31.4 ± 2.4	32.2 ± 2.7	31.3 ± 1.8	31.3 ± 23	33.0 ± 0.9	30.2 ± 3.8
$p \leq$	0	NS	NS	NS	NS	NS

Fig. 5. Plasma cortisol concentration (ng/ml) of piglets of the six groups on day 0 of the immunoprofile study

peroxidase activity of the kidneys, myocardium, brain, and skeletal muscles (Parsons et al., 1985).

In this study, the basal ration containing, on the average, 0.13 mg Se per kg was supplemented with 0.5 mg Se per kg of feed. The average daily Se intake of piglets fed the unsupplemented control diet was 0.007–0.009 and 0.004–0.006 mg/kg body mass, respectively. As a result, the mean Se concentration of their crude liver tissue was 0.45 (day 35) and 0.46 (day 76 to 83) mg/kg. With the exception of two piglets of Group 3, the higher Se concentration of the experimental diets was reflected in the higher hepatic Se concentration of the experimental piglets already in the first 5 weeks of feeding. Hepatic Se concentration of both the control piglets and the piglets fed Se--supplemented diet was in good agreement with that reported by others (Meyer



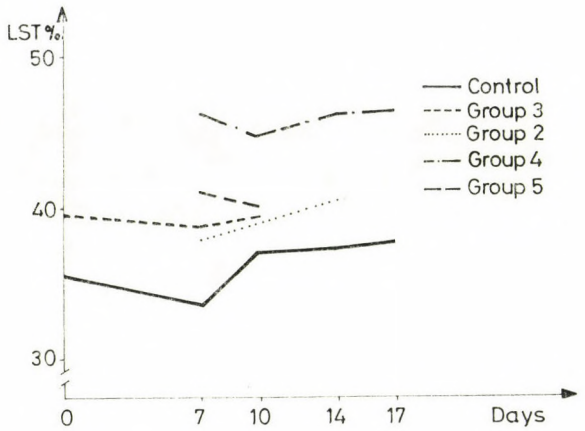
Group	Day	0	7	10	14	17
Control	n	8	8	6	6	6
	x ± SD	3.3 ± 1.5	7.2 ± 2.0	10.2 ± 1.2	16.7 ± 2.2	19.3 ± 1.4
1	n	8	7	4	4	4
	$\bar{x}$ ± SD	3.4 ± 1.4	6.7 ± 2.8	11.8 ± 1.7	12.5 ± 2.0	17.0 ± 1.0
	p ≤	NS	NS	NS	NS	NS
2	n	8	8	6	6	6
	$\bar{x}$ ± SD	3.9 ± 1.3	8.8 ± 2.1	13.3 ± 2.3	14.2 ± 2.1	19.2 ± 1.2
	p ≤	NS	NS	0.02	NS	NS
3	n	7	8	6	6	6
	$\bar{x}$ ± SD	3.0 ± 1.6	6.9 ± 2.2	10.5 ± 2.1	13.7 ± 2.6	17.7 ± 2.2
	p ≤	NS	NS	NS	NS	NS
4	n	8	8	6	6	6
	$\bar{x}$ ± SD	3.3 ± 1.0	9.4 ± 1.6	15.0 ± 1.6	16.8 ± 1.7	21.5 ± 1.9
	p ≤	NS	0.05	0.001	NS	0.05
5	n	8	8	6	6	6
	$\bar{x}$ ± SD	3.5 ± 1.4	7.3 ± 2.3	9.8 ± 2.3	15.3 ± 2.5	20.3 ± 2.2
	p ≤	NS	NS	NS	NS	NS

Fig. 6. Rate of blastogenic transformation (%) after *in vitro* stimulation of lymphocytes with horse globulin. Solid line: control; broken line: Group 4

et al., 1981; Moksnes et al., 1982), but was higher than that measured in the liver of 11- to 17-week-old piglets fed a diet supplemented with 0.5 mg Se per kg of feed (Mahan et al., 1977).

Serum vitamin E concentrations measured in blood samples taken on day 74 of feeding the experimental diets closely reflected the vitamin E quantity offered in the feed. The absolute serum vitamin E values of the control group showed good agreement with those found by Stowe and Miller (1985) from 82 and 89 days old piglets reared on a basal ration not supplemented with vitamin E. Supplementation of the basal ration with 50, 100 or 150 mg vitamin E per kg of feed induced similar changes in serum vitamin E concentration to those reported by Peplowski et al. (1981) and Jensen et al. (1983).

No correlation was demonstrable between riboflavin and vitamin E concentration of the feed and hepatic Se concentration. Hepatic Se concentra-

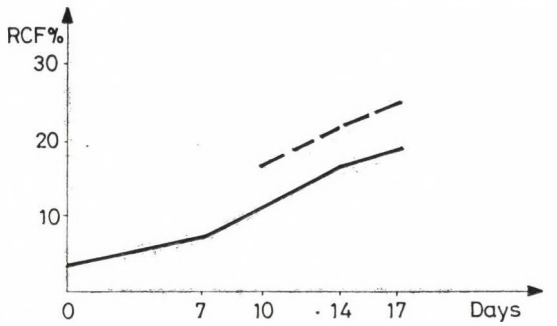


Group	Day	0	7	10	14	17
Control	n	8	8	6	6	6
	$\bar{x} \pm SD$	35.6 ± 3.7	33.6 ± 3.8	37.0 ± 1.7	37.2 ± 2.9	37.7 ± 2.7
1	n	8	7	5	4	4
	$\bar{x} \pm SD$	37.3 ± 4.5	36.7 ± 3.2	38.2 ± 2.2	37.0 ± 2.2	35.8 ± 3.5
	$p \leq$	NS	NS	NS	NS	NS
2	n	8	8	6	6	6
	$\bar{x} \pm SD$	36.3 ± 3.3	37.9 ± 2.6	41.0 ± 2.0	39.0 ± 2.6	37.0 ± 6.3
	$p \leq$	NS	0.05	0.01	NS	NS
3	n	8	8	6	6	6
	$\bar{x} \pm SD$	40.0 ± 1.7	38.7 ± 3.0	39.1 ± 1.5	38.8 ± 2.5	40.0 ± 3.3
	$p \leq$	0.02	0.01	0.05	NS	NS
4	n	8	8	6	6	6
	$\bar{x} \pm SD$	36.0 ± 4.7	46.3 ± 4.8	44.7 ± 4.7	46.0 ± 5.0	46.3 ± 5.1
	$p \leq$	NS	0.001	0.01	0.01	0.01
5	n	8	8	6	6	6
	$\bar{x} \pm SD$	32.4 ± 13.2	40.9 ± 4.3	40.0 ± 2.3	38.3 ± 2.0	37.7 ± 2.7
	$p \leq$	NS	0.01	0.05	NS	NS

Fig. 7. Rate of blastogenic transformation of lymphocytes (%) after *in vitro* stimulation with phytohaemagglutinin (PHA)

tion of the experimental piglets was higher than that of piglets reared on a diet of approximately identical Se content but unsupplemented with vitamin E and riboflavin (Mahan et al., 1977; Mahan, 1985). This fact indicates that vitamin E and riboflavin supplementation of the feed facilitated Se retention. No obvious explanation is available for the consistently high hepatic Se concentration found in Group 1.

In piglets fed a diet supplemented with 100 mg dl-alpha-tocopherol and 0.0, 0.025, 0.050 or 0.10 mg Se per kg of feed, the virtual digestibility of dry matter and nitrogen increased in direct proportion to Se supplementation



Group	Days	0	7	10	14	17
Control	n	8	8	6	6	6
	x ± SD	3.6 ± 1.1	7.1 ± 2.1	11.0 ± 1.5	16.7 ± 2.1	18.8 ± 2.3
1	n	8	7	5	5	5
	x ± SD	4.8 ± 1.04	7.7 ± 3.9	12.0 ± 3.1	17.4 ± 2.1	30.0 ± 1.6
	p ≤	NS	NS	NS	NS	NS
2	n	8	8	6	6	6
	x ± SD	4.0 ± 1.8	9.9 ± 1.7	13.8 ± 3.1	17.0 ± 1.3	18.8 ± 2.1
	p ≤	NS	0.02	NS	NS	NS
3	n	8	8	6	6	6
	x ± SD	4.8 ± 1.4	8.4 ± 2.5	13.8 ± 1.9	16.2 ± 2.1	21.2 ± 3.7
	p ≤	NS	NS	0.02	NS	NS
4	n	8	8	6	6	6
	x ± SD	3.9 ± 2.2	11.5 ± 2.3	15.7 ± 2.2	21.5 ± 2.3	24.7 ± 2.7
	p ≤	NS	NS	0.01	0.01	0.01
5	n	8	8	6	6	6
	x ± SD	4.0 ± 1.3	8.6 ± 1.7	13.2 ± 1.0	16.5 ± 1.9	19.5 ± 1.1
	p ≤	NS	NS	0.01	NS	NS

Fig. 8. Proportion of rosette-forming cells (%). Solid line: control; broken line: Group 4

(Adkins and Ewan, 1984*b*). Despite this fact, authors agree that the body mass gain, feed consumption and specific feed utilization of weaned and growing piglets cannot be influenced by changing the Se and/or vitamin E content of the diet (Piper et al., 1975; Mahan and Moxon, 1978*a*, 1978*b*; Jensen et al., 1983; Adkins and Ewan, 1984*a*; Blodget et al., 1984/1985; Mahan, 1985). Our results are fully consistent with the earlier observations, indicating that riboflavin given in excess together with Se and vitamin E also fails to exert a beneficial influence on the growth rate and feed utilization of weaned and growing piglets.

The mean plasma cortisol concentration of the control and experimental piglets was between 30.2 and 33.0 ng/ml, levels fully consistent with earlier results obtained on *ad libitum* fed piglets of similar age and reared under optimum conditions (Rafai and Fodor, 1980; Kovács et al., 1983). Se, vitamin E

Table III

Anti-horse globulin titres (log 2) at the time of the immunological profile study

Group		Day 7	10	14	17
Control	n	8	6	6	6
	x ± SD	4.63 ± 1.3	5.17 ± 0.75	5.67 ± 0.82	6.67 ± 1.03
1	n	7	5	4	4
	x ± SD	5.14 ± 1.07	5.6 ± 0.89	6.75 ± 1.5	7.75 ± 1.5
	P	NS	NS	NS	NS
2	n	8	6	6	6
	x ± SD	4.63 ± 0.92	5.67 ± 1.03	6.5 ± 0.84	7.33 ± 1.63
	P	NS	NS	NS	NS
3	n	8	6	6	6
	x ± SD	4.63 ± 0.92	5.67 ± 0.82	6.83 ± 0.98	7.67 ± 1.51
	P	NS	NS	NS	NS
4	n	8	6	6	6
	x ± SD	4.75 ± 0.46	5.83 ± 0.98	6.5 ± 1.22	7.33 ± 1.21
	P	NS	NS	NS	NS
5	n	8	6	6	6
	x ± SD	5.0 ± 1.07	5.83 ± 0.98	6.50 ± 0.84	7.33 ± 1.03
	P	NS	NS	NS	NS

and riboflavin content of the feed did not alter adrenal cortex function, as opposed to the experimental results of Watson and Petro (1982), according to which in mice the increase of dietary vitamin E content resulted in a decrease of blood plasma corticosterone concentration. Watson and Petro (1982) suggested that reduced corticosterone production was associated with the increased T lymphocyte activity measured by them.

Though the immunostimulating effect of Se and vitamin E given in excess of the requirements has been proved by numerous experiments on laboratory rodents and, of the domestic animals, on poultry, few data are available on swine in this respect. In this experiment, on days 7, 10, 14 and 17 of the immunoprofile study the anti-horse gamma globulin titres of the experimental piglets were higher than those measured in the control animals. This indicates that excess Se, vitamin E and riboflavin given in the diet favourably affect the humoral immune response of growing piglets. This finding confirms the data obtained by Ellis and Vorhies (1976) with an *Escherichia coli* vaccine in piglets, i.e. that on the effect of a daily dose of 100,000 IU vitamin E, given in excess of the requirements, in the primary immune response the HA titres of piglets immunized with formalin-inactivated *E. coli* were 2 to 3 times higher than those of the control animals. Peplowski et al. (1981) immunized piglets with red blood cell (RBC) antigen at 7-day intervals, and found that Se and vitamin E, either separately or in combination, elevated the HA titres. The result of Blodget et al. (1984/1985) is in contradiction to

data of the above authors and also to our findings. They found that supplementation of the feed with 0.3 to 1.5 mg Se per kg failed to alter the humoral immune response to lysozyme and ribonuclease given as antigen; neither did it change certain indices of cell-mediated immune response (results of the intradermal PHA test). Kruse et al. (1985) also failed to demonstrate differences between piglets fed a diet supplemented with varying amounts of vitamin E and control piglets given the basal ration, in humoral immune response elicited by 25 mg/pig bovine serum albumin as antigen.

Though in a varying degree, the feed supplementation used in this experiment exerted favourable influence on the *in vitro* indices of cell-mediated immunity tested. Both the rate of PHA-induced and horse gamma globulin induced blastogenesis and the proportion of rosette-forming cells were the highest, of the five different feeding groups, in Group 4, the group given 0.5 mg Se, 100 mg vitamin E and 5 mg riboflavin per kg of feed. In experiments with weaned piglets, Larsen and Tollersrud (1981) also found that supplementation of the feed with Se and/or vitamin E enhanced lymphocyte stimulation by PHA. Similar results were reported by Reddy et al. (1986) for calves treated with vitamin E. In the experiments of Larsen et al. (1986) with lambs, supplementation of the feed with 0.1 or 0.5 mg Se per kg feed enhanced, while that with 1.0 mg Se per kg reduced, the response of lymphocytes to stimulation with nonspecific mitogens. As opposed to the *in vitro* lymphocyte stimulation tests, the intradermal PHA reaction (a type IV allergic reaction) was not enhanced by Se, vitamin E and riboflavin supplementation. This finding seems to support the results of Blodget et al. (1984/1985).

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IMMUNIZATION OF CALVES WITH LIVE AND INACTIVATED WHOLE-CELL VACCINES AGAINST *SALMONELLA TYPHIMURIUM* INFECTION

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Eight calves were immunized with live auxotrophic *Salmonella typhimurium* mutants (aro $^-$SL 1479, gal E 3821) and twelve calves with phenol-killed whole-cell *S. typhimurium* vaccine, respectively. The clinical status of the animals was followed and serial reisolation of vaccine and challenge strains from faeces was attempted.

The immunization of calves with the live aro$^-$ auxotrophic *S. typhimurium* SL 1479 mutant proved to be unsuitable due to the death of calves after revaccination.

The calves immunized with live auxotrophic gal E *S. typhimurium* CCM 3821 mutant proved to be protected against challenge with virulent *S. typhimurium* 4/5 strain administered orally at a dose of 10^6 colony forming units (CFU). The postvaccination complications showed serious shortcomings.

The immunization of calves with three doses of whole-cell inactivated vaccine containing 5 strains of *S. typhimurium* was effective against oral challenge with virulent *S. typhimurium* 4/5 at a dose of 10^6 CFU.

Keywords: Vaccination, gal E and aro$^-$ *S. typhimurium* mutants, inactivated whole-cell vaccine, calves.

Present knowledge on the mechanisms of pathogenesis of Salmonella infections in animals and the pathogenetic factors of salmonellae leads to a critical re-evaluation of the vaccines available nowadays.

The present efforts are based upon the development of live vaccines, subcellular vaccines, inactivated whole-cell vaccines and other forms of protection against salmonellosis.

The attenuated strains for production of Salmonella vaccines are either dependent on aromatic amino acids, other aromatic compounds, antibiotics, sugars, or represent rough Salmonella mutants (Vladoianu et al., 1975; Ivanoff et al., 1982; Stocker et al., 1983; Wray and Sojka, 1984).

Subcellular vaccines, i.e. ribosomal vaccines, vaccines containing endotoxin-protein and a vaccine consisting of Boivin endotoxin are also currently tested for the purpose of immunoprophylaxis of salmonellosis (Angerman and Eisenstein, 1978).

Killed whole-cell vaccines are able to induce both humoral and cellular immune response (Nakoneczna and Hsu, 1983). The process of Salmonella inactivation is commonly carried out by heating or by chemicals such as acetone, phenol, formaldehyde, chloroform, etc. (Herzberg et al., 1972; Severtsova et al., 1979; Nauciel et al., 1981; Jaszovska, 1982; Mikula et al., 1986*a*).

Passive immunization (Jacks, 1978) and the substitution of the intestinal microflora in chickens (Baljer, 1984) are also employed in the immunoprophylaxis of Salmonella infections.

The aim of this study was to compare the efficacy of two live vaccines and one phenol-killed whole-cell vaccine against salmonellosis in calves under laboratory conditions.

Table I

Characteristics of individual strains used for preparation of inactivated vaccine

Characteristics tested	3/5	6/5	10/5	12/5	27/5
Adhesiveness (x):					
enterocytes	19.60	17.74	13.29	19.24	12.17
VERO cells	24.26	10.75	12.20	31.56	14.06
MDBK cells	23.96	13.82	12.85	19.50	14.32
Production of enterotoxin:					
skin test on rabbits	+	+	+	—	+
VERO cells	+	+	+	—	—
CHO cells	+	+	+	—	—
lig. loops of calf	+	+	+	—	—
Serum resistance (%):	20.26	15.99	240.55	1.18	474.40
Sereny test:	+(48 h)	—	+(72 h)	—	+(72 h)
Production of colicin:	—	—	—	+	—
Restriction endonuclease plasmid profile:	not identical				

Table II

Oral immunization and challenge of calves

Age of calves (days)	Group I		Group II		Group III	
	Type of vaccine	Dose	Type of vaccine	Dose	Type of vaccine	Dose
5	SL 1479 (aro⁻)	1×10^{8}	CCM 3821 (gal E)	4×10^{7}	CCM 3821 (gal E)	4×10^{7}
10	SL 1479 (aro⁻)	1×10^{10}	CCM 3821 (gal E)	4×10^{7}	CCM 3821 (gal E)	4×10^{7}
17	SL 1479 (aro⁻)	1×10^{10}	Challenged with *S. typhimurium* 4/5 at a dose of 1×10^{6}		Challenged with *S. typhimurium* 4/5 at a dose of 1×10^{9}	
26	Challenged with *S. typhimurium* 4/5 at a dose of 1×10^{6}		—		—	

Each group contains two calves

Materials and methods

Bacterial strains

The following strains of *S. typhimurium* were used in this study:

a) *S. typhimurium* SL 1479, designated as auxotropic mutant (aro-), was received from Prof. B. A. D. Stocker (USA) in 1982. This strain is avirulent to the inbred mouse line BALB/c (up to an i.p. dose of 3.5×10^6 CFU) and calves (up to an oral dose of 1.5×10^{11} CFU; Stocker et al., 1983).

b) *S. typhimurium* CCM 3821 (the gal E mutant strain used by Bioveta, Ivanovice na Hané, for production of live vaccine).

c) Five strains of *S. typhimurium* (3/5, 6/5, 10/5, 12/5 and 27/5) were obtained from the collection of our department (Table I).

d) *S. typhimurium* 4/5, a strain fully virulent for mice and calves and used for challenge.

The preparation of live and killed whole-cell vaccines

a) For the preparation of live, avirulent vaccine, *S. typhimurium* SL 1479 was grown in BHI (brain–heart infusion, Oxoid) broth for 18 h at 37 °C without shaking and aeration. After enumeration of salmonellae (by the plate-count method), the broth culture was diluted or concentrated in order to contain 10^8 or 10^{10} CFU per ml.

b) "Vaccine against salmonellosis in calves" (made by Bioveta, Ivanovice na Hané) was applied as shown in Table II.

with live *S. typhimurium* vaccines

Group IV		Group V	Group VI
Type of vaccine	Dose	Control	Control
CCM 3821 (gal E)	4×10^7	—	—
CCM 3821 (gal E)	1×10^{10} 1×10^{10}	—	—
Challenged with *S. typhimurium* 4/5 at a dose of 1×10^9		Infected with *S. typhimurium* 4/5 at a dose of 1×10^6	Infected with *S. typhimurium* 4/5 at a dose of 1×10^9
—		—	—

c) The phenol-killed whole-cell vaccines prepared from *S. typhimurium* strains 3/5, 6/5, 10/5, 12/5 and 27/5 were grown in BHI broth at 37 °C under aeration for 18 h. The bacterial strains were pooled at a mutual ratio of 1 : 1 to a final concentration of 1×10^{10} CFU. Inactivation was done with 0.5% phenol.

Animals

Calves of both sexes (5 days old) were included in this trial (Slovak red--white cattle). Each calf was kept in a separate pen and fed with milk replacer (Laktavit, commercial product) three times daily.

The immunization and challenge schedules of calves are shown in Tables II and III. Live oral vaccine was given altogether to eight calves in four groups with two calves in each. The control group consisted of four animals of which two calves were infected at a dose of 10^6 CFU and the other two with 10^9 CFU (Table II).

Inactivated whole-cell vaccine was given altogether to twelve animals of which 10 calves received three doses of this vaccine and two animals were immunized by two doses only. Six calves were included in the control group (Table III).

Table III

Intramuscular immunization of calves with an inactivated whole-cell vaccine at a dose of 1×10^{10} CFU

Group	Immunization	Age of calves (days)	Number of calves	Infection	Body temperature range after infection	Protection %	Average period of survival after infection
I	3x	2–3 7–8 14–15	10	10^6	39.1–40.8	100	Killed on day 21
II	2x	4–6 10–12	2	10^6	38.6–40.7	50	One calf died on day 3, one calf was killed on day 21
III	control	19–24	6	10^6	39.7–41.4	Ø	9 days

Oral infection on day 9 after the last immunization with *S. typhimurium* 4/5 at a dose of 1×10^6 CFU

Excretion rate of the live vaccine and challenge organisms

Attempts were made to re-isolate salmonellae from faecal samples taken from the ampulla recti every morning throughout the trial. Calves that had died were necropsied. For the enumeration of viable bacteria of the vaccine and challenge strain (CFU), samples were taken post mortem from each intestinal segment. Cultivation was carried out on Endo agar plates.

Results

Trial 1

Immunization of calves with aro⁻ auxotrophic S. typhimurium SL 1479 mutant (Table II)

Within 24 h of vaccination calves in group I developed mild illness with rectal temperature increased by 1.3 to 1.7 °C as compared to the values before vaccination. On the second day weakness and profuse diarrhoea appeared, accompanied by an increased temperature in the range of 1.6–2.7 °C over the physiological limit. The animals' health status was the same on the 4th day.

S. typhimurium strain SL 1479 was reisolated on the 1st day postvaccination (3.9×10^3 CFU per gram of faeces), while its maximum counts (7.9×10^7 CFU per gram of faeces) were detected on the 5th day p.v. Interestingly, a sudden decline of *E. coli* CFU per g faeces occurred within 1–2 days after vaccination (expressed as a decline of two orders of magnitude).

On the 5th day calves were revaccinated orally with *S. typhimurium* strain SL 1479 at a dose of 1×10^{10} CFU. The calves died within 24 h p.v. and showed gross pathological and histopathological lesions typical of salmonellosis. Salmonella was isolated from the duodenum, jejunum, ileum, spleen, liver and the mesenteric lymph nodes.

Trial 2

Immunization of calves with S. typhimurium CCM 3821 vaccine produced by Bioveta, Ivanovice na Hané (Table II)

All calves of groups II, III and IV were orally vaccinated with a dose of 4×10^7 CFU at the age of 5 days. An elevated body temperature was recorded already on the 2nd day p.v. in the immunized calves. There was a change in the consistency of the faeces of calves after vaccination, revaccination and challenge. All calves survived both vaccination and revaccination. After vaccination and revaccination, reduced *E. coli* counts were found in the faeces and *S. typhimurium* CCM 3821 was also detected.

The calves were challenged with *S. typhimurium* strains 4/5 on the 7th day after revaccination. The calves of the second group survived the challenge. One out of the two calves in group III died on the 4th day postchallenge, and both calves of group IV survived challenge with 1×10^9 CFU.

Two calves in the control group (Table II, groups V and VI) that were infected with an oral dose of 10^6 CFU died by the 12th day, and the other two calves infected with a dose of 10^9 died by the 7th day after oral administration.

Trial 3

Immunization of calves with phenol-killed whole-cell vaccine (Table III)

Ten calves in group I received phenol-killed vaccine in three doses given intramuscularly (each dose contained 1×10^{10} CFU in 2 ml) on the 5th and 12th day after the first vaccination. A slight elevation of body temperature was recorded in calves after the vaccination, revaccination and challenge, respectively. No local reaction was found after intramuscular application. The calves survived challenge with a dose of 1×10^{6} CFU. Diarrhoea was not observed in any of the calves during the whole 21-day period. The challenge strain was reisolated from faeces on the 2nd, 3rd and 12th day after infection. One out of the 2 calves of group II survived the challenge. An elevated body temperature was recorded in calves after challenge. *S. typhimurium* challenge strain 4/5 was rarely recovered between days 8 and 10 from the single calf that survived postchallenge. In the other calf that died 5 days after challenge, *S. typhimurium* 4/5 counts reached values of 3.2×10^{2} to 4.5×10^{3} CFU per gram of faeces.

After the 21-day postchallenge period, calves were killed and necropsied. The intestinal tract and parenchymal organs were examined microbiologically. During this investigation, the challenge strain was reisolated from the internal organs, i.e. from the ileal lymph nodes and/or the walls of ileum and large intestine but not from the liver and spleen.

The microbiological findings obtained for the control group (Table III, group III) were similar to those of the control calves (Table II, group V).

Discussion

S. typhimurium G 30D mutant, a strain belonging to the gal E group, is often used as live vaccine against salmonellosis. Germanier (1972) concluded that vaccination with live gal E mutant gave rise to a better protection.

A vaccine prepared by Wray et al. (1977) was effective in conferring protection on mice, calves and poultry challenged with high doses of *S. typhimurium*.

Our results obtained on 5 days old calves vaccinated with live *S. typhimurium* gal E mutant are essentially consistent with the results of Wray et al. (1983).

Bairey (1978) reported protection of three to six weeks old calves after using the formaldehyde-killed whole-cell vaccine containing $Al(OH)_3$ gel and administered subcutaneously.

On the other hand, Smith et al. (1980) achieved good protection by immunization with live virulent *S. typhimurium* strains.

Solid immune response was obtained by Aitken et al. (1982) after intradermal application of two doses of heat-killed *S. dublin* organisms. However, these authors did not take into account the possible age-related resistance against Salmonella infection.

Heat-inactivated vaccine adsorbed on $Al(OH)_3$ gel was prepared by Robertson et al. (1983). The average survival period of calves after the challenge was 21 days. Similarly to our experiments, the challenge strain was recovered from the faeces already during the first 5 days postchallenge.

We believe that pathogenicity factors (Table III) should be taken into consideration in the production of vaccine (Mikula and Pilipčinec, 1983; Mikula et al., 1986*b*).

The vaccine prepared in our laboratory proved to be effective in the model experiment in three vaccinations. In our opinion its use in field outbreaks of salmonellosis could enable us to find strains with new characteristic individual properties. Isolates obtained from vaccinated calves will be subjected to a study of pathogenicity factors and provide an opportunity for vaccine improvement.

The presented results indicate that the drawbacks of the live vaccine strain were due to the following adverse effects:

1) In immunized animals the postvaccination reaction appeared in altered values of TBP (fever, increased respiratory and heart rate) and in the consistency of faeces (semi-solid or liquid, profuse diarrhoea).

2) Faecal shedding of vaccine strain during the postvaccination period (Clarke and Gyles, 1986; Mikula, 1988).

3) The potential risk of transmission of virulent plasmids cannot be excluded after vaccination (Baljer, 1984).

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ADAPTATION OF 5'-DEIODINATION TO HYPOTHYROID CONDITIONS FOLLOWING SURGICAL AND/OR RADIOTHYROIDECTOMY IN CHICKENS*

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The effect of thyroidectomy on serum levels of thyroid hormones (thyroxine, T4; triiodothyronine, T3) and on tissue levels of T3 as well as on 5'-deiodination (5'-DI) in liver, kidney and brain of chickens was investigated.

The most effective way of thyroidectomy was a combined surgical (Tx) plus radiothyroidectomy (RTx) with low amounts of 131-I (NaI). The latter destroyed extra-thyroidal hormone production.

It was found that even if after Tx and RTx the serum levels of T4 and T3 were close to the detection limit, the tissue T3 level was only half of normal in liver and kidney and remained normal in the brain. It is suggested that lowered 5'-deiodination is an important but not exclusive factor contributing to this adaptation of cells to hypothyroid conditions.

Keywords: Surgical thyroidectomy, radiothyroidectomy, thyroxine, triiodothyronine, 5'-deiodination, chicken.

Thyroidectomy has widely been used in the chicken *(Gallus domesticus)* for different experimental purposes. First of all, thyroid physiology can readily be studied when this endocrine gland is extirpated and replacement studies give important information about the production rate of thyroid hormones (Mellen and Wentworth, 1962). Secondly, the role of the thyroid in animal production as well as in the maintenance of endocrine balance in correlation with other endocrine organs could better be studied in the thyroidectomized chicken as a model (Astier et al., 1978).

With the specific radioimmunoassay for thyroxine (T4) and triiodothyronine (T3), determination of the serum level of these hormones became possible. As it was summarized recently by others (Harvey et al., 1983) and shown also by this research group (Rudas and Pethes, 1980; Péczely et al., 1980), the concentration of thyroid hormones in the serum of thyroidectomized chickens did not disappear totally and in most of the cases it remained well detectable even weeks after thyroidectomy.

The presence of thyroid hormone in the blood serum after thyroidectomy can be explained by several factors. First of all, in some cases one should pre-

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sume a failure to remove the whole gland. A second reason may be the activation of some embryonically dispersed stem cells, as proposed by Nalbandov (1974) in mammals. The presence of non-hormonal immunoreactive substance should also be investigated in the bird (Astier and Newcomer, 1978).

Also the role of the binding proteins, namely the changes in their ability to bind T4 and T3, may contribute to the phenomenon. Since it has been proved (Rudas and Pethes, 1984) that the conversion of thyroxine to triiodothyronine is more extensive in chickens than in rats, and also that hypothyroidism can affect thyroid hormone activation in rats (Silva et al., 1984), it is of interest to investigate the involvement of peripheral deiodination in shaping serum and tissue levels of thyroid hormones after thyroidectomy.

Materials and methods

Experimental animals

Hunniahybrid chickens were used throughout. The birds were 1–2 weeks old when transferred to the laboratory from the farm. A 1-week adaptation period was applied in the case of controls. Surgical thyroidectomies were done some days after arrival. Room temperature was kept at 23 °C, water and food were available *ad libitum*. Continuous lighting (24 L, O D) was applied in order to eliminate diurnal fluctuations.

Thyroidectomies (Tx)

Surgical thyroidectomy was carried out under Nembutal (25 mg/kg body mass) narcosis. Both the right and the left glands were removed without damaging any of the airsacs. The wound was sutured with only 2–5 stitches. Oxytetracycline (Tetran pulvis, Chinoin, Budapest) was used to prevent bacterial growth.

Thyroidectomy was considered to be complete when both the right and the left organs could be removed in their unhurt capsules.

All thyroidectomies were followed by blood sampling for basal levels of thyroid hormones.

The effectiveness of surgical thyroidectomy was checked by determining the serum level of thyroid hormones (see later) and also by postmortem investigation of the area of the original location of the glands.

Histological examination (haematoxylin–eosin staining) was also carried out in several cases where visual inspection could confirm no thyroid remnants. In experiment 2 histological investigation of this area was done after repeated thyrotropin (bovine TSH, Sigma Chem. Co., St. Louis) treatment, the aim of which was to stimulate the growth of putative remnants of the original thyroid

tissue. Another way to check the remnants was the injection of 131-I (NaI) intravenously. In these trials the accumulation of 131-I in the neck area could be detected but no specific rise of activity was confirmed on the original site of the gland. This spoke in favour of no operational mistake.

Radiothyroidectomy (RTx)

Carrier-free 131-I (NaI) of 1200 Ci/mmol specific activity was used in all experiments. Animals were injected intraperitoneally with 600 μCi of the 131-I, in 0.5 ml saline/100 g body mass. Controls were injected with saline alone. All samples, taken before and during this operation, were deep frozen for more than 3 months (below —20 °C) in order to eliminate 131-I activity. This period (more than 10 times of the half-life of the 131-I) allowed that the radioactivity of the samples (either blood or organs) did not interfere with any of the determinations used. This was confirmed in all analytical procedures, but detailed description is not given here.

Determination of T4 and T3 plasma concentrations

Highly specific radioimmunoassay was used for the determination of T4 and T3 (Pethes et al., 1978*a*, *b*). The intraassay variance of the determinations ranged between 5–7 per cent. Although the interassay variance was also under 10 per cent in all the series of measurements, for a more precise comparison, samples from parallel experiments were processed in the same assay run. The sensitivity of the assay is of major importance here: the detection limit was 0.168 nmol/l and 1.285 nmol/l for T3 and T4, respectively. Cross reactivity of the antisera used in this experiment with other iodothyronines was low enough not to disturb the measurement even in the lowest concentrations (Rudas, 1983).

T3 content of tissue samples

The concentration of T3 in the tissue samples was determined by the alcoholic extraction procedure described by Nejad et al. (1975). Extraction efficiency ranged from 85 to 90% for T3 (Rudas, 1983). The tissue level measurements were validated by the following method. 125-I–T3 was injected intravenously and the tissue to serum ratio (T/S) of T3 was determined at the maximum of the tissue uptake curve. (Paper chromatography was used to make sure that only the 125-I activity coming from labelled T3 and not from other metabolites be considered.) This ratio was then used to calculate an estimate of the tissue T3 concentration using the serum T3 value obtained by RIA. The results of such estimates agreed with the tissue T3 RIA within 10% (coefficient of variation).

5'-deiodinase assay

The activities of liver, brain and kidney deiodinases were determined according to the method described earlier (Scanes et al., 1983) and modified slightly later (Rudas, 1986). Briefly, it entailed the determination of the 5'-deiodinase (type I) enzyme activity by measuring the T3 produced from known amount of T4 added by tissue homogenates. Results are expressed as pmol T3 produced/mg tissue protein/h. Protein determinations were carried out according to the method described by Lowry et al. (1951).

Experiment 1

The aim of this experiment was to compare the effectiveness of two types of thyroidectomies. Surgical thyroidectomy was performed on 15 chickens from which ten were selected on the basis of whether the capsule of the removed gland was intact. Another group of 10 birds were injected with 131-I (600 μCi/100 g body mass). Blood samples were taken immediately before and 4, 7, 12, 17 and 21 days after the injection of 131-I.

Experiment 2

This trial was performed to check whether the hormone producing cells existing two weeks after surgical thyroidectomy are responsive to thyrotropin. Bovine thyrotropin (bTSH; 200 μg/100 g body mass, Sigma Chem. Co., St. Louis) was used in ten 7-week-old surgically thyroidectomized chickens 19, 25 and 30 days after thyroidectomy to check the responsiveness of serum thyroxine level upon bTSH treatment, in comparison to ten nontreated, thyroidectomized birds. Blood samples were taken regularly as indicated in "Results".

Experiment 3

The activity of the liver and kidney 5'-deiodinase as well as the tissue level of triidothyronine were observed in this experiment. Three groups of birds were formed. Group I served as control (C). Group II involved chickens thyroidectomized surgically (Tx) and group III consisted of birds thyroidectomized surgically and injected with a low dose of 131-I, that is with 300 μCi/100 g body mass intraperitoneally. This was done on the basis of a former experiment (Veresegyházy et al., 1986) where this combination was found to be the least stressing and most effective thyroidectomy (RTx). Twenty birds in each main group (I, II, III) were divided into subgroups of 10 birds each.

Subgroups "a" were untreated controls, subgroups "b" were treated with iopanoic acid (IOP), a contrast agent known as a complete blocker of 5'-deiodination (10 mg/100 g body mass) 24 and 12 h before killing the chickens. The ability of this drug to block deiodination in the chicken was checked in a

pilot experiment, where 100 mg IOP/1000 g body mass was given per os to birds in groups of three individuals each. Liver 5'-deiodination was then measured in these birds 0, 4, 8, 16 and 24 h after administration of IOP. Seven days after Tx and 14 days after RTx, on the day of the experiment the birds were killed, blood, liver, kidney and brain (without pituitary and cerebellum) samples were taken and kept frozen until determinations. Livers were carefully washed out with 0.9% NaCl (4 °C) through the vena portae in order to empty the tissue of blood serum. Regular blood sampling was also carried out during the experiment so that the serum levels of thyroid hormones could be followed throughout.

Statistical analysis included comparison of groups by means of Student's *t*-test.

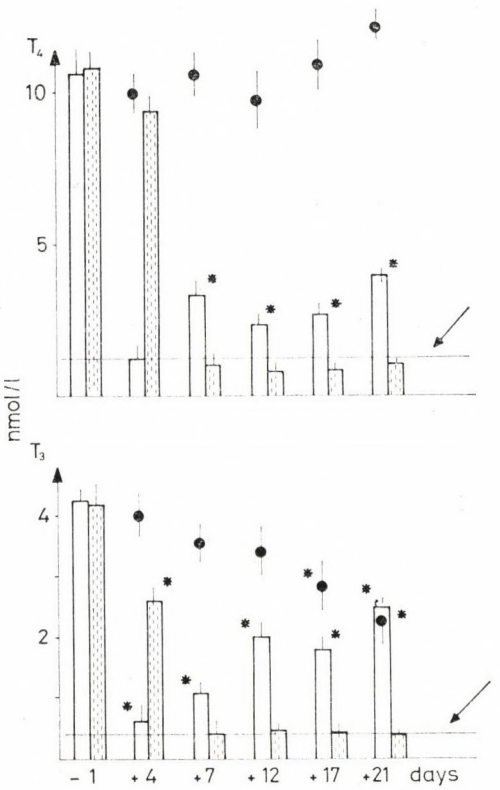

Fig. 1. The effect of surgical (open bars) and radiothyroidectomy (shaded bars) on the serum levels (nmol/l) of thyroxine (T4) and on that of triiodothyronine (T3) in two-week-old chickens one day before (−1) and several days (+4–+21) after thyroidectomies. Radiothyroidectomy was performed using 600 μCi 131-I-(NaI)/100 g body mass ip. Arrows show detection limits (+2 standard deviation from "0" standard). Dots above or on the bars represent normal, non-thyroidectomized values of serum T4 and of T3 at the respective age. Latter values are taken from a former longitudinal study on the same breed of chickens (Rudas, 1983) and are given here for comparison. ($\bar{x}\pm$SEM; N = 8–10; *P < 0.05 vs. −1 day; no statistics were made on groups at or below detection limit)

Results

Measuring serum concentration of thyroid hormones before and after surgical or radiothyroidectomy in Exp. 1, one could observe detectable hormone levels after surgical intervention, but no thyroid hormones after radiothyroidectomy (see Fig. 1).

It appears from Fig. 1 that 600 μCi 131-I/100 g body mass destroys the thyroid of birds totally. In further experiments with chickens (Exp. 3) Tx + 300 μCi 131-I was selected to achieve total thyroidectomy without applying too high doses (Veresegyházy et al., 1986).

It is obvious from the data that low level of immunoreactive thyroid hormone is present in surgically thyroidectomized, but not in radiothyroidectomized birds. Triiodothyronine levels seem to normalize after about three weeks while this is not the case with T4.

It should be noted, however, that in some cases the "undetectable" hormone level in radiothyroidectomized birds seems to be on the borderline of the sensitivity of the assay. Note also that even if the overall intraassay variance of the RIA procedures applied is about 5–7 per cent, it is the nature of this analytical method that at both ends of the standard curve (high and low concentrations) one has to count with higher error.

The further investigation of the nature of immunoreactive hormonal "remnants" in the serum was carried out in Exp. 2. If no TSH treatment was

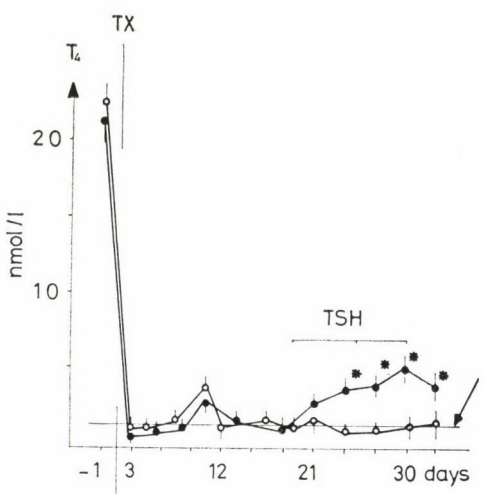

Fig. 2. Two groups of 7-week-old chickens, 10 birds in each, were surgically thyroidectomized in this experiment. On day 19, 25 and 30 after thyroidectomy (TX) one group (—●—) was injected with bovine thyrotropin (TSH; 200 μg/100 g body mass, im.). The other group (—○—) served as control. The level of thyroxine (T4) in the blood serum was measured by RIA. Detection limit (+2 standard deviation from "0" standard) is indicated by an arrow. (nmol T4/L serum; x±SEM; N = 8–10; *P 0.05 —○— vs. —○—)

done (Fig. 2), T4 concentrations averaged 1.0–1.5 nmol/l vs. 21.4 ± 1.8 nmol/l in non-thyroidectomized birds. However, when regular treatment with bTSH was carried out, a significant increase of serum thyroxine concentration could be detected (Fig. 2).

Serum concentration of thyroid hormones in Exp. 3 for Tx, RTx and C animals was found to be similar to that demonstrated in Fig. 1 for Exp. 1 (results not shown here).

The effect of surgical (Tx) and combined surgical + radiothyroidectomy (RTx) on the tissue levels of the active thyroid hormone, that is of T3, is shown in Fig. 3. While serum concentrations of thyroid hormones are close to the detection limit (see above), the tissue concentrations are depressed only to the half of the original level of sham-operated controls (C). This is the case with liver and kidney. The level of T3 in the brain remains at the pre-thyroidectomy concentration.

It can be seen that iopanoic acid that blocks T4 to T3 conversion completely (see later) has no further depressing effect on the tissue level of T3 when administered to thyroidectomized (either Tx or RTx) animals. However, it is a potent suppressor of the tissue concentration of triodothyronine in normal, non-thyroidectomized controls. It is of interest that the decreased

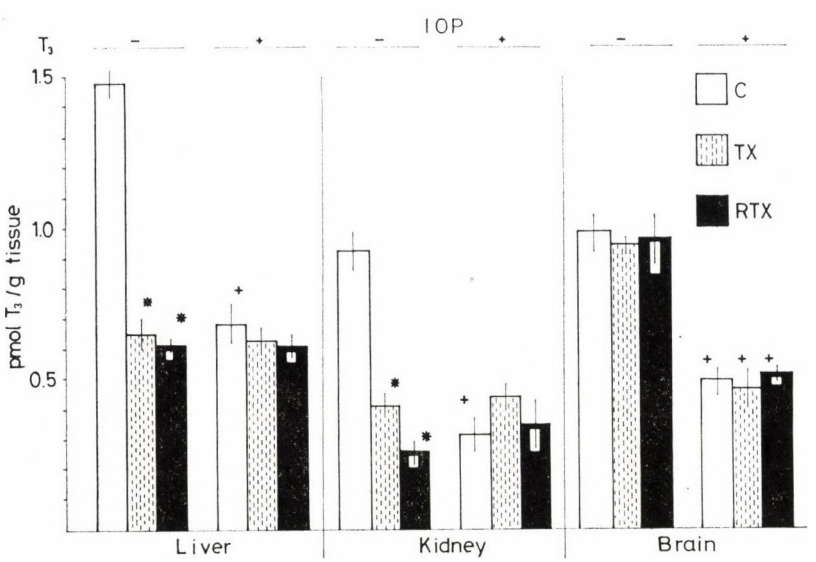

Fig. 3. Tissue levels of triiodothyronine (T3) in three groups of chickens. C = controls; Tx = = surgically thyroidectomized at 4 weeks of age; RTx = surgically thyroidectomized at 3 weeks of age followed by 300 μCi 131-I–(NaI)/100 g body mass ip. All birds were sacrificed on the same day at 5 weeks of age. IOP + = birds in these groups were treated with 10 mg iopanoic acid (IOP)/100 g body mass 24 and 12 h before sacrificing them. IOP — = no IOP treatment. (pmol T3/g fresh tissue; x±SEM; N = 8–10; *P < 0.05 vs. respective control; $^{+}$P < 0.05 IOP+ vs. IOP—)

tissue concentration achieved by *in vivo* administration of IOP equals that found in thyroidectomized animals.

The activity of the tissue 5'-deiodinase was measured both in the presence (10 mM) and in the absence of the cofactor (DTT). Results are shown in Fig. 4. The original enzyme activity, that is the non-stimulated enzyme activity (DTT) of the liver and kidney of thyroidectomized chickens, was significantly lower than that found in controls. Roughly, both ways of thyroidectomies caused a halving of the original enzyme activity. When the cofactor (DTT) was added *in vitro* to the system, the activity of 5'-deiodinase in the liver and in the kidney of thyroidectomized animals reached the catalytic activity of the controls. Blocking of the enzyme activity *in vivo* by giving IOP caused a dramatic drop in the liver, kidney and brain 5'-deiodination of all groups and there was no significant influence of thyroidectomy on this inhibition.

The brain tissue (that contained the whole brain except pituitary and cerebellum) distinguished itself from the other two tissues both in its response to thyroidectomy and in sensitivity to DTT. The 5'-deiodinase activity in the brain was found not to be influenced by thyroidectomy and it was not stimulated by the cofactor, DTT. *In vivo* treatment with IOP was able to depress brain levels of T3 to the half of the original concentration.

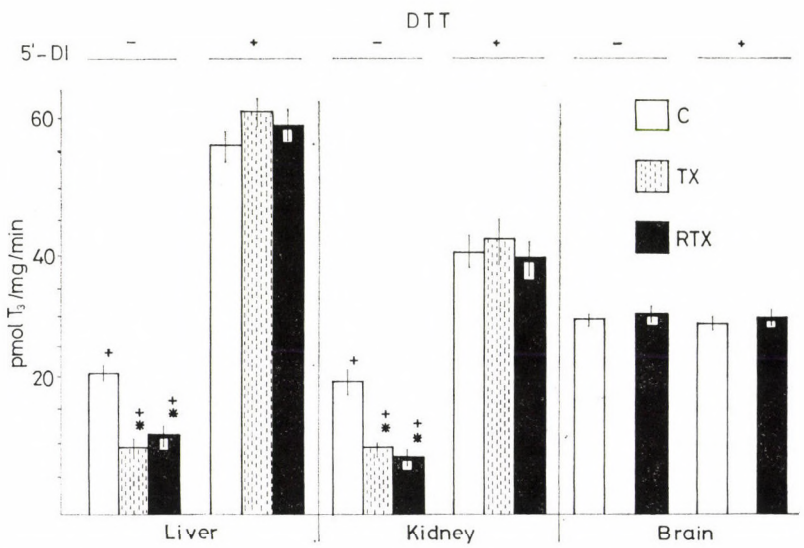

Fig. 4. The activity of 5'-deiodinase (5'-DI) in three groups of chickens. C = controls; Tx = = surgically thyroidectomized at 4 weeks of age; RTx = surgically thyroidectomized at 3 weeks of age followed by 300 μCi 131-I-(NaI)/100 g body mass ip. All animals sacrificed on the same day at 5 weeks of age. DTT + = enzyme activity measured in the presence of 10 mM dithiothreitol (DTT); DTT − = no DTT added. The enzyme activity is expressed as the amount of T3 produced from exogenously added T4 by tissue homogenates per unit of time (pmol T3/mg/min = pmol T3 produced/mg of tissue/minute; x ± SEM; N = 8–10; *P < 0.05 vs. respective control; +P < 0.05 DTT + vs. DTT−)

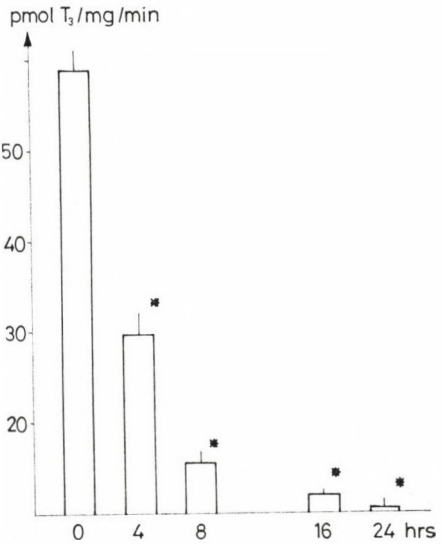

Fig. 5. Pilot experiment on the effectiveness of orally administered iopanoic acid (IOP) on the activity of liver 5'-deiodinase (5'-DI) in 4-week-old chickens. 10 mg IOP/100 g body mass was given at time "0". The 5'-DI was measured as the amount of triiodothyronine (T3) produced from exogenously added thyroxine by liver homogenates. (pmol T3 produced by mg of liver per one minute; x±SEM; N = 6; *P < 0.05 vs. "0" time)

The time dependence of the effect of IOP in the pilot experiment showed that 24 h post-treatment there was an almost total (>95%) blocking of 5'-deiodination (Fig. 5).

Discussion

The question of whether surgical thyroidectomy and/or radiothyroidectomy can yield totally athyreotic animals has been raised from time to time. In relation to the chicken the first observation of athyreotic conditions after radiothyroidectomy (Wentworth, 1949) was thoroughly reinvestigated by Mellen and Wentworth (1962). The dose they applied was equal to the highest dose (1200 μCi/100 g body mass) injected in one of our former experiments (Veresegyházy et al., 1986). In contrast to our results, however, almost no loss of birds due to radiothyroidectomy was seen by Mellen and Wentworth (1962). It was concluded that if radiothyroidectomy is complete, no hormonal activity can be detected in the serum of birds. This speaks in favour of our experiment but the final conclusion that can be drawn from the comparison of surgical and radiothyroidectomy does not confirm the view of the above-mentioned authors. In our opinion the presence of low levels of thyroid hormones in the serum of surgically thyroidectomized birds takes its origin not from the thyroidal remnants of unsuccessfully operated birds but as a result of other facts.

Provocation with bTSH stimulated the T4 level in the serum of surgically thyroidectomized chickens. The increase was significant; however, it was less than that found in normal chickens in previous studies (Rudas, 1983). This speaks in favour of our former supposition, namely that extrathyroidal thyroid hormone producing activities may exist. Since no thyroid remnant was found either with inspection or with histology on the area from which the glands were removed, we believe that the source of the hormonal activity might be originated either from dispersed embryonic cells on the neck area as proposed by Nalbandov (1974), or from iodinated albumin from the blood pool as suggested by Shulman et al. (1957). The fact that after radiothyroidectomy the level of thyroid hormones was closer to or below the detection limit might suggest that iodinated proteins either from internal or external (food) origin play a minor role in replenishing the disappearing blood thyroid hormone pool after thyroidectomy.

Another fact is a change in the peripheral handling and thyroidal metabolism of thyroid hormones by the tissues as an effect of thyroidectomy. This is strongly supported by the present results.

First, T3 levels normalized after three weeks of thyroidectomy but T4 levels did not. This refers to an important shift in the overall T4 → T3 conversion in order to attain close to normal T3 concentrations.

Second, the most important finding in our present experiments is that the tissue level of thyroidectomized birds decreased to a far less extent than serum levels. Beside the excellent agreement of this fact in birds with findings of Obregon et al. (1981) in rats, it raises the possibility of a preserving mechanism functioning in thyroidectomized animals which might involve a series of factors one of which is the lowered intensity of the 5'-deiodination of the thyroxine available. To this latter mechanism a series of others join, e.g. the decreased clearance, i.e. increased residence time of triiodothyronine within the cells. As can be concluded from tissue levels of triiodothyronine, there exists a well defined lowest limit of T3 concentration that is maintained by the organism for a very long time after thyroidectomy. It is of interest that this portion of intracellular T3 (about 50% of the normal intracellular T3 level) will not disappear from the cell even a long time (70 days, authors' unpublished observation) after thyroidectomy. It is known from studies on rats (Larsen and Silva, 1983) that about this portion of intracellular T3 pool is maintained by deiodination in that species. Since it was proved that IOP is a potent inhibitor of T4 to T3 conversion in the chicken also (Decuypere et al., 1982; Rudas and Pethes, 1984; and results in Exp. 3, as well as the pilot experiment on IOP action) and since the level of intracellular T3 is depressed to the same degree by *in vivo* administration of IOP as is by thyroidectomy, one can suggest that the decreased liver and kidney 5'-deiodination is a major but not single factor in the adaptation to hypothyroidism of the iodothyronine deprived

cells. The extent of the deiodinase-independent portion of intracellular T3 pool is similar to that found in rats (Silva et al., 1984). Since the enzyme activity present in thyroidectomized chickens can readily be stimulated by DTT, one has to presume that the impaired availability of reducing equivalents in hypothyroidism, which results in a lower level of SH groups, is involved as a major cause in the decreased 5'-deiodination. Otherwise, the synthesis and thus the intracellular concentration of the enzyme is probably not influenced by thyroidectomy. Recent publications (Larsen and Silva, 1983; Visser et al., 1983) called attention to a new type of 5'-deiodinase, which distinguishes itself from the type-I enzyme in its relative insensitivity to the blocking effect of PTU (type II).

The brain distinguished itself with no response to thyroidectomy as for tissue level of T3 and 5'-DI activity. The concentration of triiodothyronine maintained at the pre-thyroidectomy level will gain full explanation only when, beside deiodination activity, the clearance rate of T3 from brain cells will be estimated. Attempts should be made also to differentiate between the alterations in enzyme populations within the brain after thyroidectomy. It might be that behind unchanged 5'-DI-type I activities other deiodination routes may change their activity.

IOP treatment depressed original tissue T3 levels to the same degree in the brain as was seen in the liver and in the kidney. This further supports the view that pathways of deiodination are involved in the adaptation of brain cells to hypothyroidism, even if there must be a series of other factors contributing to this phenomenon.

It was found that supplementation of the incubation medium with DTT enhanced deiodination activity in liver and in kidney homogenates but at the same time the difference of 5'-DI activity between thyroidectomized and control birds disappeared. This fact not only supports the view that the amount of the enzyme present in the cell might be normal (see above) but calls the attention to a methodological problem as well. Namely, that the routine method of investigating deiodination in the presence of stimulator (DTT) might cover up important details. It is suggested therefore that the low but informative 5'-DI activities in the absence of the stimulator should also be regarded. A similar conclusion has been drawn from rat studies recently (Leonard et al., 1983).

The authors believe that these findings, together with several recently published results in the literature (for review see Silva et al., 1984), further support the view that the deiodinase system and the peripheral handling of thyroid hormones represent a special autoregulative defense mechanism against either hypothyroidism or other environmental influence when the demand on available thyroid hormones is altered (Rudas and Pethes, 1980).

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RESTRICTED FEED INTAKE INFLUENCES THYROID HORMONE PRODUCTION AND PERIPHERAL DEIODINATION IN CHICKENS

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Thyroid hormones are of major importance in determining metabolic state both in mammals and in birds. Whether or not feed intake itself has an autoregulatory role in adjusting the setpoint of thyroid hormone activity is yet not well understood. The present work investigates the effects of restricted feed intake on the serum levels and on the peripheral metabolism of thyroid hormones. Also, the sensitivity of the pituitary–thyroid axis was looked at by means of thyrotropin releasing hormone provocation test. Several groups of Hunniahybrid chickens aged 2–6 weeks were used. Restriction was made by providing the experimental animals 70 and 85 percent of the amount of food consumed in the *ad libitum* fed control group. It was found that feed restriction lowers circulating concentration of triiodothyronine probably by inhibiting the activity of liver deiodinase and also decreases the sensitivity of the pituitary–thyroid axis meaning that both central and peripheral regulatory mechanisms of thyroid economy are affected by feed restriction.

Keywords: Thyroxine, triiodothyronine, deiodination, energy intake, domestic fowl.

One of the best known effects of thyroid hormones is that of influencing metabolic state. It is generally accepted that the active hormone in this respect is triiodothyronine that firmly bounds to nuclear and other cellular receptors and exerts thermogenic action mainly by increasing futile cycles of energy expenditure (Ismail-Beigi et al., 1979). The last decade has brought a tremendous amount of information regarding the intricate mechanism by which the availability of triiodothyronine for the individual cells is regulated. In the centre of this mechanism stands the deiodinase enzyme that can convert thyroxine produced by the thyroid gland either to active triiodothyronine or to inactive reverse-triiodothyronine, depending on the actual needs of the organism (Silva and Larsen, 1985). These phenomena are true also for domestic birds (Decuypere et al., 1981; Rudas and Pethes, 1984; Hughes and McNabb, 1986). Among a series of factors that might influence this system is the availability of energy equivalents to the cells. This might be of great importance in animal nutrition. The aim of the present paper was to demonstrate that the amount of food ingested affects thyroid economy and the knowledge of this influence might change our view about optimal feeding conditions.

Materials and methods

Forty-five Hunniahybrid chickens were raised in groups of 5 birds in each cage. From day one to week four of age chickens were provided with feed and water *ad libitum* from automatic troughs. Lighting regime (16 hours light: 8 hours dark) and temperature control (age-dependent room temperature) was set by an automated time switch and thermostat, respectively. On day 28 of age the birds were allotted to three major groups (3×5 birds): control (100%), feed intake restricted to 70% of control (70%) and feed intake restricted to 85% of control (85%). The amount of food provided to the experimental groups was determined on the basis of the daily food consumption of the control group. On day 32 of age (4th day of feed restriction) blood samples were taken from 5 birds in each group. This was followed by an iv. injection of thyrotropin releasing hormone (TRH) (5 μg/100 g body mass). Exactly 30 min thereafter another blood sampling took place from the contralateral brachial vein. Another 5 individuals from each group were bled by cervical dislocation. Liver was taken out from these birds and was deep frozen until determinations. The above procedure was repeated on day 40 of age (12th day of feed restriction) and on day 47 of age (19th day of feed restriction). The remaining 15 birds were subjected to blood sampling and the TRH provocation test was carried out on day 19. The next day the same birds were bled and livers were processed as above.

Thyroxine (T4) and triiodothyronine (T3) concentrations in the blood serum and in homogenates were determined according to the radioimmunoassay procedure described formerly for chicken (Pethes et al., 1978). The logit-log procedure suggested by Rodbard and Lewald (1970) was used for the computer program that evaluated RIA results. The deiodination activity of the liver was determined according to our former procedure (Scanes et al., 1983) as modified recently (Rudas and Pethes, 1986). In short, this procedure involves the homogenization of the liver sample in phosphate buffer (pH 7.4, 0.15 M) followed by gentle (1000 *g*, 4 °C, 10 min) centrifugation. The supernatant is incubated with known amount of T4 (0.67 μM) and the T3 produced after 60 min is determined following an alcoholic precipitation. Cofactor (dithiothreitol, DTT) is also present (8 mM). The results are expressed as T3 produced/hour/g liver protein.

The results of the TRH provocation test are given as the percentile increase in serum T4 concentration caused by the single intravenous injection of 2 μg/100 g body mass TRH.

Statistical analysis entailed Student's *t*-test for comparison of groups. Differences were accepted as significant if P was less than 0.05.

Results

Pooled data of the different time points (4th, 12th, 19th day of feed restriction) corresponded to the results obtained on the separate experimental days. Thus except for deiodination, pooled results are shown here.

Table I summarizes the results of serum T4 and T3 concentrations for the two experimental and for the control group. Separate data for the different time points were similar. T3 serum concentrations are significantly lower, while T4 levels are consistently higher in feed restricted animals.

Table I

Concentration of triiodothyronine (T3) and thyroxine (T4) in the blood serum of chickens after feed restriction (ng/ml)

Available food (in per cent of the *ad libitum* group)		70%	85%	100%
T3	average	0.608***	0.591***	1.296
	±SEM	0.076	0.081	0.138
	N	15	14	15
T4	average	18.28***	19.58***	7.43
	±SEM	2.02	2.16	1.43
	N	15	15	13

*** $P < 0.01$ vs 100%

The results of the TRH provocation test are summarized in Table II. The experimental groups (70% and 85%) exhibit almost full insensitivity to TRH stimulus. 85% feed restriction is almost as effective as 70% in this respect.

Table II

The effect of TRH provocation test upon plasma level of T4 after feed restriction (% change versus baseline T4 level)

Available food (in per cent of the *ad libitum* group)		70%	85%	100%
% increase in T4 serum level 30 min after i.v. TRH inj.	average	28.5***	39.4***	160.2
	±SEM	9.83	11.3	38.9
	N	14	15	15

*** $P < 0.01$ vs 100%

The pooled data of the deiodinative activity of liver samples (Table III) reveals no significant difference among the groups investigated. However this parameter was not identical during the whole experiment. The lower panel of this table shows that when data from the 4th day of feed restriction are sepa-

Table III

Activity of liver 5'-deiodinase after feed restriction in chickens (pmol T3 produced/hour/g liver)

Available food (in per cent of the *ad libitum* group)		70%	85%	100%
pooled data	average	20.6	20.6	21.7
	±SEM	1.91	2.85	2.39
	N	15	14	14
4th day of feed restriction	average	16.8*	19.7	23.2
	±SEM	1.93	2.54	4.86
	N	5	5	5

* $P < 0.05$ vs 100%

rated from the pooled values then a significantly lower deiodination rate could be confirmed in the 70% group versus the 100% control. Deiodination rate is also lower in the 85% group, however this difference is not statistically significant.

Table IV

Body mass of chickens used in the experiments as measured at different time points (g)

Available food (in per cent of the *ad libitum* group)		70%	85%	100%
4th day	average	891	960	910
	±SEM	44.9	73.1	68.3
	N	5	5	5
12th day	average	1300	1304	1495
	±SEM	51.8	49.1	50.1
	N	5	5	5
19th day	average	1238**	1422	1602
	±SEM	70.5	61.6	91.1
	N	5	5	5

** $P < 0.02$ vs 100%

Table IV contains data on the body mass of the different groups of animals. It is obvious from this table that body mass differences accumulate and become significant at the end of the test period.

Discussion

On the basis of feeding experiments it was postulated by Klandorf et al. (1981) that liver deiodination might be a possible trigger for a systematic drop of serum T3 concentration seen after feed restriction. The above results speak in favour of the above supposition. It is clear from the data that as

soon as 4 days after feed restriction liver 5'-deiodination diminishes significantly.

Data presented here are in accordance with the findings of Harris et al. (1979) who demonstrated that the increase in the availability of energy equivalents results in a NADPH dependent increase of the hepatic deiodinase activity in rats, and also with the data that demonstrate a similar pattern for the domestic hen (Decuypere and Kuhn, 1984). The increased activity of the liver to convert T4 to T3 is one of the explanations for the increased triiodothyronine level observed in the serum of chickens in the 100% group. When restriction of feed intake occurs there is another consequence of the lowered uptake of thyroxine by the liver (and probably kidney) cells: serum T4 concentrations rise due to lack of uptake by these sites or, as postulated by others, due to the presence in the plasma of some undetermined metabolites that interact with T4 (Chopra et al., 1979). The insensitivity of the pituitary–thyroid axis to TRH provocation is due to the elevated serum T4 concentrations. This high level of T4 in the blood is a negative feed back signal probably via the pituitary type-II deiodinase system (Silva and Larsen, 1985) in the rat. In chickens it was shown recently (Bartha et al., submitted for publication) that pituitary deiodinases behave similarly and can contribute to rhythmic changes in the serum level of thyroid hormones. On the basis of the results one might suppose that thyroidal T4 output is also decreased in feed restriction. The controversial high level of T4 in the serum of the feed restricted groups is due to a balance existing between T4 production and degradation: it should be supposed that T4, even if produced at a lower rate, elevates in the serum since the decrease in its degradation exceeds the decline in its production.

It was seen that deiodination is lower in the feed restricted groups on day 4, but later on (day 12 and 19) the difference disappears. This fact suggests that the regulatory role of deiodination might occur earlier than the investigated period. Other investigations concerning the factors affecting deiodination in the chicken have already called attention to the very quick response of this regulatory mechanism. Changes in environmental temperature may elicit a response of the deiodination system as early as 4 h after mild cold exposure in the chicken (Rudas and Pethes, 1980). Whether or not such a rapid response is also elicited by feed restriction remains to be answered.

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EFFECT OF INTRARUMINAL BUTYRATE INFUSION ON THE PLASMA INSULIN LEVEL IN SHEEP

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After intraruminal infusion of butyrate to sheep at dose rates of 0.25, 0.5, 1 and 2 g sodium *n*-butyrate per kg body mass, butyrate concentration of the rumen fluid and total secreted insulin rose in direct proportion to the butyrate dose infused. The half-life of butyrate in the rumen was always longer than that of insulin. At 90 min after the infusion of 1 g butyrate per kg body mass, butyrate concentration in the ruminal papillae reached the level corresponding to an extracellular concentration that reduced cell division by 50% *in vitro*. It can be concluded that butyrate may be present in the ruminal papillae in concentrations inhibiting cell proliferation, simultaneously with the presence of blood plasma insulin concentrations stimulating the proliferation of ruminal epithelial cells.

Keywords: Sheep, intraruminal infusion, butyrate, cell division, ruminal epithelial cells, insulin.

In ruminants, the epithelium lining the rumen has numerous physiological functions including transportation of volatile fatty acids (VFA) produced in the rumen. Undisturbed and satisfactory absorption requires an optimally developed and sufficiently thick ruminal epithelium. The status and number of strata of the ruminal epithelium depend on the division rate of the cells constituting it.

Earlier *in vitro* studies (Gálfi et al., 1981) have shown that *n*-butyrate decreases the division rate of ovine ruminal epithelial cells (REC). For these cells, the extracellular concentration of butyrate that reduces cell division by 50%, is 0.2 mmol/l (Gálfi et al., 1988).

However, according to Sakata and Tamate (1976) and our experiments (Gálfi et al., 1986), in the case of rapid intraruminal infusion *n*-butyrate increases the division rate of REC *in vivo*. It probably exerts this effect through a mediator or mediators. One of the possible mediators is insulin (Sakata et al., 1980). This suggestion is supported by the observation that insulin secretion in ruminants is enhanced by elevated butyrate concentrations in the blood (Brockman, 1978).

In our earlier *in vitro* experiments insulin was found to enhance DNA synthesis of REC both in 20 and in 200 μU/ml concentrations. If butyrate and insulin were present in the medium simultaneously, in different concentrations, the cell division rate depended on the ratio of these two compounds (Neogrády et al., 1989).

To elucidate the effect of butyrate and insulin on ruminal epithelium *in vivo*, the changes of the butyrate concentration of the rumen fluid, ruminal papillae and plasma, as well as of plasma insulin level, were determined in sheep given a single intraruminal infusion of butyrate.

Materials and methods

Animals and feed. Five Merino ewes (no. 1–5) of 35 to 40 kg body mass, having a permanent rumen fistula, were used. Prior to the experiments the animals had received alfalfa hay *ad libitum* and 300 g concentrate daily, in two parts.

Treatment. On the evening before the day of intraruminal infusion of sodium *n*-butyrate, feed was withdrawn from the animals. The next morning, at the time of the feeding, a catheter was inserted into the left jugular vein. Half an hour later 0.25, 0.5, 1 and 2 g sodium *n*-butyrate per kg body mass was infused into the rumen of sheep 1–4, respectively, in 10% aqueous solution. Sheep no. 5 served as control. After the infusion the rumen content was thoroughly mixed through the fistula. Sheep no. 3 received another infusion of 1 g sodium *n*-butyrate per kg body mass a week later.

Sampling. Immediately before and 15, 30, 45, 60, 90, 120, 150, 180 and 240 min after butyrate infusion samples were taken from the rumen content and the jugular vein. In one case (sheep no. 3, second infusion) samples were taken only 90 min after infusion from the rumen content, ruminal mucosa, portal and jugular vein. Ruminal mucosa samples were taken through the fistula from four different sites (cranial blind sac, ventral and lateral part of the ventral sac, dorsal sac) on each occasion.

Laboratory tests. Rumen fluid samples were purified by filtration and acidification (Couenotte et al., 1979). The plasma samples obtained from heparinized blood were prepared according to the method of Húsvéth and Gaál (1987).

The excised rumen mucosa pieces were divided in two parts: one was homogenized in PBS (+4 °C, 3000 rpm, 15 min) and centrifuged at +4 °C with 5000 rpm for 15 min. The supernatant was purified further in the same way as the plasma, and was finally used for determination of butyrate concentration. The other half of the rumen mucosa sample was weighed, dried at 105 °C until constant mass, and the butyrate concentration of the ruminal papillae was related to the mass unit determined in this way.

The samples (rumen fluid, ruminal papillae, plasma) were assayed for *n*-butyrate concentration with a CHROM-42 gas chromatograph fitted up with a flame-ionization detector.

Blood glucose concentration was measured immediately after blood sampling by an enzymatic method (Glucostrat test, Gödecke).

Plasma insulin concentration was determined with an insulin-RIA kit (Institute of Isotopes, Hungarian Academy of Sciences, Budapest) using ovine insulin (Sigma) as standard. The results were calculated and tested by linear and exponential regression analysis and by trapezoid and Simpson methods (insulin response areas above baseline concentrations between 0 and 240 min) (McCann and Reimers, 1985*b*). Exponential removal-rate equations for concentrations of injected butyrate in rumen and secreted insulin in plasma were calculated from the regression equations. The antilog value of the y-axis intercept was substituted for A_0 (concentration of insulin in plasma at time zero), the slope of the regression line was substituted for K_E (fractional removal rate constant) (McCann and Reimers, 1985*a*). Biological half-life (t 1/2) was calculated as 0.693: K_E by Ritschel's method (1980).

Results

Ruminal butyrate and plasma insulin concentrations following butyrate infusions are shown in Fig. 1. (Values obtained after the infusion of 0.5 g/kg *n*-butyrate are shown only in Table I.) Butyrate concentration in the rumen content was considerably elevated at the first determination (5th min) after

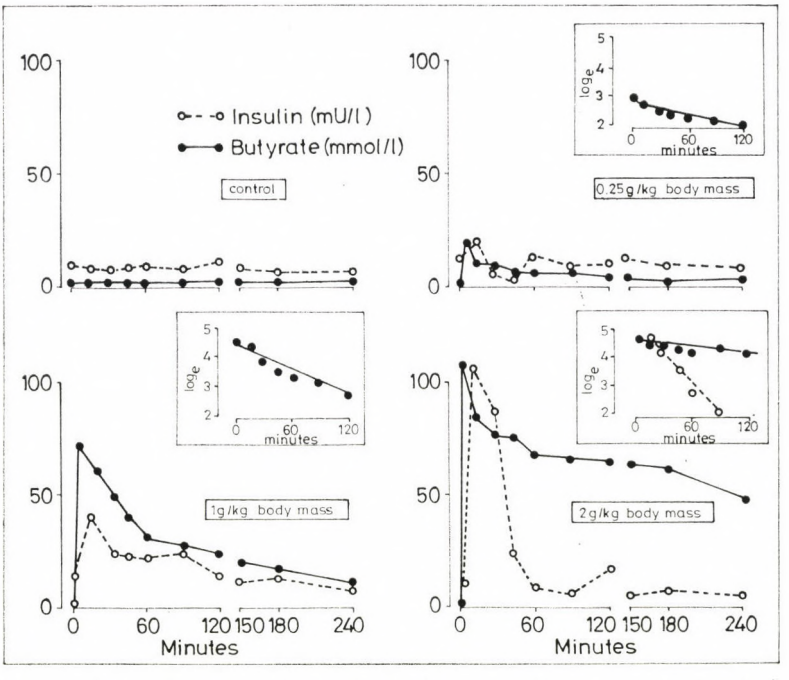

Fig. 1. Butyrate concentration of the rumen fluid and insulin concentration of the plasma after intraruminal infusion of *n*-butyrate

Table I

Removal rate of sodium *n*-butyrate from the rumen and secretion of insulin after intraruminal infusion of sodium *n*-butyrate

Sodium n-butyrate (g/kg body mass)	0	0.25	0.5	1.0	2.0
A_0* (mmol/l)	—	18.3	47.02	80.28	105.57
K_E** (min^{-1})	—	0.013	0.014	0.016	0.009
t 1/2*** (min)		54.9	50.7	42.5	78.7
Secreted insulin (μU/ml/min)	7.1	11.9	11.3	22.5	30.6

*, **, *** concentration of *n*-butyrate in the rumen fluid
A_0 = concentration of butyrate at time 0 (y-axis intercept of the removal rate curve)
K_E = removal rate constant
t 1/2 = biological half-life

the infusion and it gradually decreased thereafter. Plasma insulin concentration was also substantially elevated at 15 min after the infusion (20–120 mU/l) and subsequently it declined rapidly. Plasma insulin concentration always returned sooner to the pre-infusion values than did butyrate concentration in the rumen content. The "inserts" show the log-transformed concentrations: for insulin, only values obtained after the 2 g/kg dose are shown, since only these could be fitted to the regression line.

The characteristics of the regression lines and the total secreted insulin are shown in Table I. The A_0 value of butyrate concentration was directly correlated with the butyrate dose infused ($r = 0.9489$). K_E decreased only

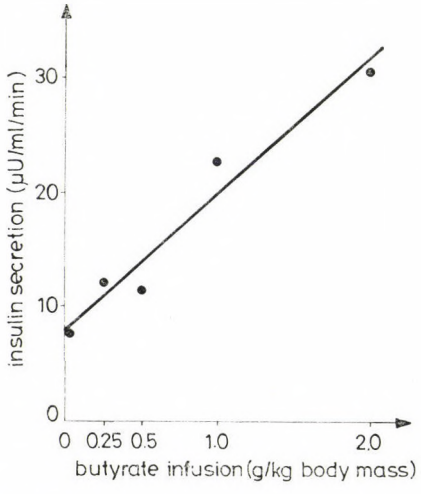

Fig. 2. Correlation between the infused *n*-butyrate dose and total insulin secreted between 0 and 240 min (n = 5)

after the 2 g/kg dose: after lower doses of *n*-butyrate it was nearly identical. T 1/2 slightly decreased up to a dose of 1 g/kg, whereas it increased after the 2 g/kg dose. After the infusion of 2 g/kg *n*-butyrate the A_0, K_E and t 1/2 of the removal rate equation of secreted insulin was 181.3 μU/ml, 0.040 min and 17.3 min, respectively (results not shown in Table I). Total secreted insulin was in linear correlation with the dose of butyrate infused. There was a close correlation (r = 0.9774) between insulin secreted (0–240 min) and the concentration of infused butyrate (Fig. 2).

Blood glucose concentrations are shown in Fig. 3. As compared to the pre-infusion values (2.0–2.9 mmol/l), taken as 0 for easier representation, at 15 min a mean elevation of about 0.5 mM, while at 30–60 min a mean decrease of the same value was demonstrated. At later samplings blood glucose concentrations were found similar to the pre-infusion values.

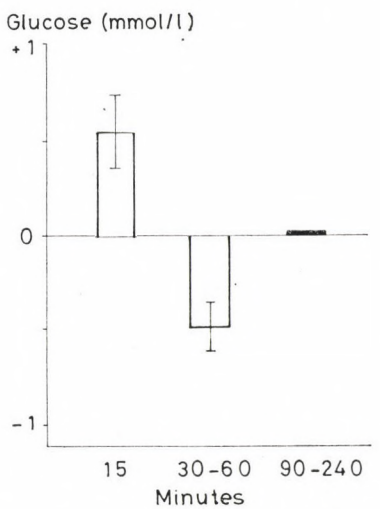

Fig. 3. Blood glucose concentration after intraruminal infusion of *n*-butyrate. The value shown as 0 actually represents an initial glucose concentration of 2.0 to 2.9 mmol/l (n = 4; $\bar{x} \pm SD$)

Table II

Butyrate concentration in the rumen fluid, ruminal papillae and plasma 90 min after intraruminal infusion of 1 g sodium *n*-butyrate per kg body mass (sheep no. 3)

n butyrate (mmol/l)			
rumen fluid	ruminal papillae+	portal vein	jugular vein
43.69	0.25 ± 0.08[a] (0.72 ± 0.23)*	4.65	0.04

+ mean of samples taken from four different sites of the ruminal mucosa
[a] mmol/l ($\bar{x} \pm SD$)
* μmol/g tissue ($\bar{x} \pm SD$)

Table II shows the butyrate concentrations in the rumen fluid, ruminal papillae and plasma measured 90 min after the second 1 g/kg butyrate infusion (sheep no. 3). In the rumen fluid butyrate concentration was 43.69 mmol/l, while in the ruminal papillae a much lower concentration (0.25 mmol/l) was measured. Plasma butyrate taken from the portal vein was 4.65 mmol/l, and that from the jugular vein was found to be as low as 0.04 mmol/l.

Discussion

In this experiment, the removal rate of butyrate from the rumen correlated with the infused dose of butyrate. The decrease of K_E and the increase of t 1/2 after the infusion of 2 g/kg butyrate suggested that this concentration exceeded the butyrate-eliminating capacity of the rumen. The A_0, K_E and t 1/2 values of the removal rate of secreted insulin from blood were determined after the infusion of 2 g/kg butyrate. K_E was high and t 1/2 (17.3 min) was about 22% of that of butyrate. After an infusion of 20 to 200 mU/kg, the t 1/2 of insulin in cattle was reported to be 5 to 13 min (McCann and Reimers, 1985*a*), which is in good agreement with the values measured by us in sheep.

Considering the t 1/2 of butyrate in the rumen fluid (42.5 to 78.7 min), it appears that the removal of butyrate from the rumen fluid takes longer time than that of insulin from the plasma. As REC possess specific insulin--binding receptors (Neogrády et al., 1987), the shorter t 1/2 measured in the blood does not necessarily mean that insulin cannot exert its action on the cells, since insulin molecules may be bound to receptors of REC for a long time.

As total secreted insulin was found to be closely correlated with the butyrate dose administered (as opposed to blood glucose concentration which does not show a close correlation either with infused butyrate or with secreted insulin), it seems probable that, for a certain amount of time, butyrate absorbed from the rumen into REC and insulin bound to the receptors of cells may act simultaneously on REC.

In this respect, in our earlier experiments it was found that, in certain concentrations, insulin may affect cell division also in the presence of butyrate. Two μmol/ml butyrate decreased the division rate of REC to one-third *in vitro*. When, on the other hand, 2 μmol/ml butyrate and 20 μU/ml insulin were present simultaneously in the medium, the intensity of cell division was the same as that of the control cells, i.e. insulin neutralized the cell division inhibiting effect of butyrate (Neogrády et al., 1989).

As a substantial proportion of butyrate is metabolized in the ruminal papillae, it was of interest to monitor the actual butyrate concentration in the ruminal papillae after butyrate infusion and to determine whether butyrate concentrations capable of inhibiting the division of REC were present *in vivo*.

At 90 min after infusion of 1 g/kg butyrate the extracellular plus intracellular butyrate concentration of ruminal papillae was 0.25 mmol/l. According to the results of our earlier studies, this butyrate concentration inhibits cell division *in vitro*, as an extracellular butyrate concentration of 0.2 mmol/l was shown to inhibit REC division in 50% (Gálfi et al., 1988).

From these results it appears that butyrate and insulin concentrations capable of influencing the division of REC may be present in the ruminal papillae and in the blood, respectively, also simultaneously. The time-course of the elevation of butyrate and insulin levels depends on the feeding conditions and the type of microbial fermentation. This, in turn, determines the combined and separate effects of the two substances on REC division.

Acknowledgement

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EFFECT OF HEAT TREATMENT AND SUBSEQUENT UREA SUPPLEMENTATION OF SUNFLOWER MEAL ON THE *IN VITRO* RUMINAL DEGRADABILITY OF CRUDE PROTEIN CONTENT AND ITS POSTRUMINAL DIGESTIBILITY

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A three-phase laboratory procedure was used for predicting the degradability of the protein of extracted sunflower meal before (SFM) and after heat treatment (HSFM). The rumen fluid degraded 69.1% and 67.1% of the SFM and HSFM protein, respectively. The digestibility values of rumen undegraded protein (UDP) were 57.3% (SFM) and 57.0% (HSFM) with pepsin and 17.6% (SFM) and 15.9% (HSFM) with pancreatin. Urea supplementation practically did not alter the rumen degradability of HSFM protein, while the pepsin digestibility of UDP decreased to 47.2%.

Four fractions (NH_3, dissolved amino acids, oligopeptides and proteins) of rumen degradable crude protein (RDP) were also determined *in vitro:* 81 to 92% of the degraded crude protein was found in the fractions tested. Heat treatment reduced free NH_3 content but did not alter the other three fractions. Urea supplementation decreased the quantity of NH_3, peptides and proteins as well.

Keywords: *In vitro*, protein degradability in rumen, digestibility, extracted sunflower meal, heat treatment, urea supplementation, digestion of crude protein, crude protein fractions, rumen degradable protein.

For their tissue metabolism, ruminants require amino acids absorbable from the small intestine, in varying quantities depending on their physiological status and intensity of production. Proteins getting into the rumen with the feed will be transformed by the catabolic and anabolic processes going on there. This fact offers substantial advantage for ruminants if the biological value of the protein entering the rumen is low. At such times the newly synthesized microbial protein may meet the host animal's requirements better than dietary protein does. The protein synthesized by the rumen microorganisms, however, meets only the requirements of maintenance, slow growth, gestation and late lactation (Chalupa, 1975; Kempton et al., 1977). Therefore, during rapid growth and early lactation ruminants need protein sources that escape degradation in the rumen and, after postruminal digestion, serve directly for supplying the host animal with amino acids (rumen undegradable protein UDP). Matters are further complicated by the fact that mirobial proteins

contain certain amino acids essential for ruminants only in low concentrations. These amino acids can be supplied either in the form of protected amino acids (Bhargava et al., 1977; Lundquist et al., 1985) or with proteins that escape degradation in the rumen (Ørskov, 1982). Natural feeds also contain UDP. Their UDP content can be increased by different physical or chemical treatments. One of the most widely used procedures is heat treatment by which the proportion of UDP can be increased (Schingoethe and Ahrar, 1979; Broderick and Craig, 1980; Kung et al., 1983; Madsen and Hvelplund, 1985).

There are many data in the literature on the rumen degradability of untreated extracted sunflower meal (SFM) (Setala et al., 1984; Veresegyházy et al., 1984; Madsen and Hvelplund, 1985), but our knowledge is scarce as regards the protein degradability of heat-treated extracted sunflower meal (HSFM) and the characteristics of the rumen degradable protein fraction. In the present experiments, therefore, besides the *in vitro* protein degradability of SFM and HSFM, some crude protein (CP) fractions of the degraded protein were also determined. Furthermore, the effect of urea supplementation on the degradability of HSFM proteins and on the crude protein fractions of the degraded protein were also studied.

Materials and methods

The commercially available SFM (crude protein content: 424 g/kg feed) was used as untreated feed. The first step of the production process was a mechanical dehulling. This was followed by heating up to 70–80 °C, extrusion,

Table I

Composition and nutritive value of the daily ration of sheep

Ingredients	Groups		
	I	II	III
Ground corn grain, kg	1.07	1.07	1.07
SFM*, kg	0.25	—	—
HSFM**, kg	—	0.25	0.25
Alfalfa hay, kg	0.20	0.20	0.20
Wheat straw, kg	0.10	0.10	0.10
Mineral, g	16.00	16.00	16.00
Urea, g	—	—	9.00
Specifications:			
Dry matter intake, kg	1.454	1.454	1.463
Digestible energy intake, MJ	20.94	20.94	20.94
Crude protein intake, g	219.0	217.0	243.0
Ca intake, g	4.1	4.1	4.1
P intake, g	3.3	3.3	3.3

* SFM = Sunflower meal
** HSFM = Heat-treated sunflower meal

then chemical extraction at 40 °C. Solvent residues were removed by toasting, and finally the extracted sunflower seeds were ground. The protein was briefly exposed to a temperature of 100 °C during the manufacturing process.

SFM was heat treated in a laboratory exsiccator at 120 °C for 45 min, in a drying basket (30×30×25 cm) lined with filter paper. The basket, which contained 500 g SFM at a time, was suspended on a rack and placed in the exsiccator. Heat transmission by conduction was thus prevented and uniform heat treatment of the about 3 cm thick feed layer was ensured. During the heat treatment the crude protein content of SFM decreased to 417 g/kg feed. Before the *in vivo* determinations the particle size of the test material was adjusted to 1 to 2 mm by sifting it twice. Rumen fluid was obtained from four mature Hungarian Worsted Merino wethers fitted up with a chronic rumen fistula. The animals received SFM (Group I), HSFM (Group II) and HSFM + urea (Group III) in a Latin square arrangement (Table I). For the *in vitro* studies the rumen fluid was collected after a 10-day adaptation period.

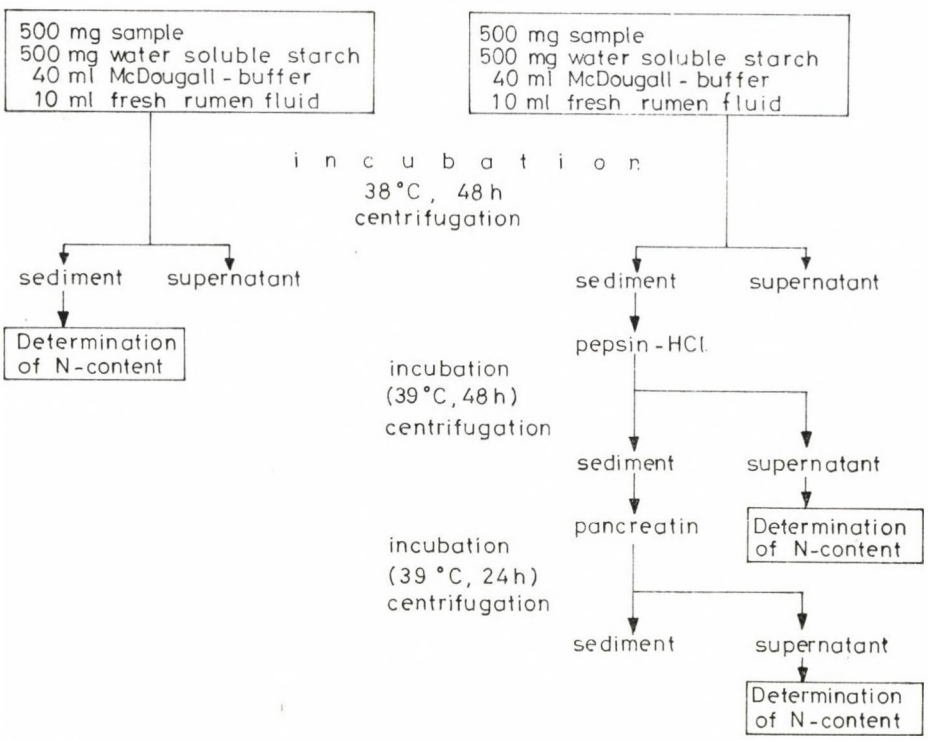

Fig. 1. The scheme of the three-phase *in vitro* method for the estimation of protein degradability and digestibility in ruminants

Digestibility experiments

In vitro digestion was done by the three-phase laboratory method described by us earlier (Veresegyházy et al., 1987; Veresegyházy et al., 1989). The principle of the method is shown in Fig. 1. In the first phase, 5×2 incubations were done with the rumen fluid of each animal, using 500 mg test material per incubation. Urea was dissolved in McDougall's buffer (1948) in a concentration of 1.25 g urea/l buffer. Each measurement series included a blind test (incubation without test material). At the end of the 48-h incubation period, digestion was stopped by adding 5% $HgCl_2$ solution, and the mixtures were centrifuged. From half of the centrifuged samples (5 parallels) the supernatant was discarded and the sediments were digested further with pepsin and pancreatin in the second and third phase, respectively. The sediments of the other half of the samples (5 parallels) were used for determining rumen undegraded protein (UDP), while their supernatants served for determining the crude protein fractions of rumen degradable protein (RDP).

Determination of crude protein content

For UDP determination, crude protein was measured in the first-phase sediments, while for determining the pepsin and pancreatin digestibility of UDP it was measured in the supernatants from the second and third phases. The determinations were performed with a Kjell-Foss instrument, in the presence of mercuric oxide as catalyst, potassium sulphate to raise the boiling point, and hydrogen peroxide as oxidizer. Crude protein was obtained by multiplying then N content by 6.25. For the lack of homogeneity the entire sediment was used up.

Determination of the crude protein fractions of RDP

With 1 ml of the phase one supernatants the biuret reaction was performed to estimate total dissolved peptide plus protein quantity. Bovine serum albumin of different concentrations, dissolved in 0.9% NaCl, was used as standard solution.

2.5 ml of the phase one supernatant was deproteinized with the same volume of 10% trichloroacetic acid. After centrifugation the supernatant was used for the biuret reaction, Sörensen's formol titration (Sós, 1974) and ammonia determination by Berthelot's method (Keller et al., 1967).

Oligopeptide concentration was determined by the biuret reaction in the deproteinized supernatant. The quantity of dissolved protein was calculated from extinction differences between the initial and the deproteinized supernatant.

The concentration of free amino acids was obtained by multiplying by 120 (mean molecular mass of amino acids) the moles of the NaOH used up in formol titration.

Ammonia concentration was determined by Berthelot's method. To 0.2 ml of the phase one deproteinized supernatant 2 ml solution A* and 2 ml solution B** were added. After shaking them thoroughly, the mixtures were incubated at 37 °C for 15 min and the intensity of the blue colour reaction was measured by photometry at 550 nm wavelength. $(NH_4)_2SO_4$ solution of 10 mg/ml concentration was used as standard solution.

Results

Digestibility of crude protein

Crude protein digestibility was obtained as the difference between the initial material and the phase one sediment in crude protein content. The individual values of crude protein measured in the phase one sediments are given

Table II

The crude protein and UDP[1] content of SFM[2], HSFM[3] and HSFM + U[4]

Sample	Crude protein g/kg	Animals			
		1	2	3	4
		Undegradable protein g/kg			
SFM[2]	424	127	104	118	155
		89	115	164	128
		94	167	118	136
		135	128	154	145
		161	108	118	152
HSFM[3]	417	106	130	153	183
		114	165	159	175
		141	186	230	200
		176	101	175	192
		183	127	154	144
HSFM + U[4]	417 (HSFM)	159	156	195	127
		156	137	132	241
	291 (Urea)	135	125	168	121
		155	111	166	117
	708	150	173	161	186

[1] UDP = Rumen undegradable protein
[2] SFM = Sunflower meal
[3] HSFM = Heat-treated sunflower meal
[4] HSFM + U = Heat-treated sunflower meal with urea supplementation

* Solution A: dissolve 5 g phenol and 25 mg sodium nitroprusside in 500 ml distilled water

** Solution B: dissolve 2.5 g NaOH and 5 ml cc. HOCl in 500 ml distilled water

Table III

In vitro digestibility of UDP[1] content of SFM[2], HSFM[3] and HSFM + U[4]

Crude protein fraction of RDP[5]	Item	Animals				Mean	% of crude protein
		1[a]	2[a]	3[a]	4[a]		
		mg/g					
RDP[5]	SFM[2]	303	299	290	281	293	69.1
	HSFM[3]	273	275	243	238	257	61.6
	HSFM + U[4]	266	277	253	259	264	63.3
UDP[1]	SFM[2]	121	125	135	143	131	30.9
	HSFM[3]	144	142	174	179	160	38.3
	HSFM + U[4]	151	141	164	158	154	36.9
Pepsin · HCl digestible UDP[1]	SFM[2]	75	75	75	75	75	17.7
	HSFM[3]	80	100	100	85	91	21.8
	HSFM + U[4]	90	100	45	55	73	17.5
Pancreatin digestible UDP[1]	SFM[2]	11	33	24	25	23	5.4
	HSFM[3]	17	21	29	36	26	6.2
	HSFM + U[4]	29	22	22	22	24	5.8
Total digestible UDP[1]	SFM[2]	86	108	99	100	98	23.1
	HSFM[3]	97	121	129	121	117	28.1
	HSFM + U[4]	119	122	67	77	96	23.0

[1] UDP = Rumen undegradable protein
[2] SFM = Sunflower meal
[3] HSFM = Heat-treated sunflower meal
[4] HSFM + U = Heat-treated sunflower meal with urea supplementation
[5] RDP = Rumen degradable protein
[a] each value represents 5 examinations

in Table II. The three test materials significantly differed in mean RDP and UDP content (Table III). As in the HSFM only 61.6% of the crude protein was degraded, in HSFM + U and SFM this value was 63.3 and 69.1%, respectively. The UDP quantities used for further digestibility studies in the second and third phase were as follow: 131 (SFM), 160 (HSFM) and 154 (HSFM + U) g/kg dry matter. Heat treatment resulted in a significant rise of the UDP content ($P < 0.01$ and $P < 0.05$ for HSFM and HSFM + U, respectively).

The *digestibility of UDP with pepsin* was nearly the same for SFM and HSFM (57.3 vs. 57.0%). However, because of the bigger UDP quantity this meant significantly more digested protein in the case of HSFM (75 vs. 91 g/kg dry matter). This index was the lowest for urea supplementation (HSFM + U; 47.2%), but for HSFM + U the quantity of pepsin-digested UDP was nearly identical with that obtained for SFM (75 vs. 73 g/kg dry matter).

The *digestibility of UDP with pancreatin* was nearly the same for all three protein sources (17.6%, 15.9% and 15.3% and 23.1, 25.4 and 23.5 g/kg dry matter for SFM, HSFM and HSFM + U, respectively). The quantity of total digestible UDP was the biggest for HSFM, but its proportion within UDP was almost the same for HSFM and SFM (73.0 vs. 74.9%). Due to its poorer

Table IV

Crude protein fractions of RDP[1] after *in vitro* incubation

Crude protein fraction of RDP	Item	Animals				Mean ± SEM (n = 20)
		1	2	3	4	
		mg CP[2]/g feed				
Dissolved proteins	SFM[3]	68	36	26	40	43 ± 4.8
	HSFM[4]	36	25	47	25	33 ± 5.0
	HSFM + U[5]	5	46	41	20	28 ± 5.2
Dissolved oligopeptides	SFM[3]	34	19	34	30	29 ± 2.5
	HSFM[4]	33	34	16	13	24 ± 2.5
	HSFM + U[5]	14	11	0	23	12 ± 3.2
Free amino acis (μmol×120)	SFM[3]	94	124	123	113	114 ± 8.6
	HSFM[4]	118	146	119	150	133 ± 8.8
	HSFM + U[5]	75	163	75	104	104 ± 14.9
Ammonia-N×6.25	SFM[3]	84	108	66	86	86 ± 4.4
	HSFM[4]	64	9	72	40	46 ± 5.8
	HSFM + U[5]	34	66	81	95	69 ± 5.8
Sum of different crude protein fractions	SFM[3]	280	287	249	269	271
	HSFM[4]	251	214	254	228	237
	HSFM + U[5]	128	286	197	242	213

[1] RDP = Rumen degradable protein
[2] CP = Crude protein
[3] SFM = Sunflower meal
[4] HSFM = Heat-treated sunflower meal
[5] HSFM + U = Heat-treated sunflower meal with urea supplementation

digestibility with pepsin, the total digestible UDP of HSFM + U was lower (62.5%); however, because of the high standard deviation this difference did not prove significant.

Crude protein fractions of RDP

Eighty-one % (HSFM + U) and 92% (SFM and HSFM) of the rumen degradable protein (RDP) could be recovered in the four fractions tested (Table IV). The single heat treatment decreased only the ammonia N content significantly (by 46 and 20%; $P < 0.01$). Urea supplementation did not cause a substantial change in free amino acid concentration but it did a significant decrease the other three fractions (dissolved protein and ammonia: $P < 0.05$; dissolved peptides: $P < 0.001$).

Discussion

Extracted sunflower meal, which is obtained in large quantities as a by--product of oil industry, may have an important role in the protein supply of ruminants. The amino acid composition of its rather high (33 to 44%) protein

content is favourable, but most of this protein is degraded in the rumen. Microbial proteolysis was estimated at 72–81% (Jarrige, 1978), 73% (Madsen and Hvelplund, 1985) and, depending on the feeding level, 55 to 82% (ARC Supplement, 1984). These data were mainly obtained by an *in situ (in sacco)* method. In this study a similar value (69.4%) was obtained by an *in vitro* method. Santos and Figueredo Nunes (1984) found the *in vitro* degradability of crude protein to be 77%. In our study, the digestibility of the UDP fraction with pepsin and pancreatin was high (57.3 and 17.6%, respectively), and thus 92.2% of the total crude protein was degraded during the three phases of the digestion applied.

The rumen degradability of the different feeds can be influenced by heat treatment. Depending on its duration, heat treatment could reduce the rumen degradation of cottonseed meal protein even to 50% (Broderick and Craig, 1980). The originally 60% rumen degradability of soybean meal protein could be reduced to a level as low as 18%, depending on the temperature used (Madsen and Hvelplund, 1985). Although during the processing of sunflower seeds their protein in exposed to heat for a short time, this heat exposure seems to be insufficient to cause protein denaturation. This is supported by the observation that a 45-min exposure to 120 °C significantly increased the proportion of UDP (from 30.9% to 38.3%). Heat treatment did not affect the digestibility of UDP with pepsin and pancreatin (57.0% and 15.9%, respectively), thus in the second and third phase, due to the bigger UDP quantity, more protein was transformed to peptides or free amino acids absorbable from the small intestine.

Though crude protein digestibility of HSFM (RDP + total digestible UDP) was somewhat, but not significantly, lower (89.6%) than that of the untreated test material, heat treatment markedly increased the quantity of digestible protein reaching the small intestine (from 98 mg/g feed to 117 mg/g feed; Table III). Urea supplementation practically did not change the UDP content of HSFM. Though ammonia-N derived from urea splitting is an easily accessible substrate for microbial protein synthesis, the higher ammonia concentration did not reduce the rate of proteolysis. This observation is consistent with results of the *in vivo* experiments of Rohr et al. (1983). Bacterial protein synthesized from urea, which would have increased the UDP fraction, was distinguishable from UDP of the test material by adding urea to the blind test. The mean crude protein content of the blind tests ($n = 8$) made from the rumen fluid of animals fed untreated and heat-treated extracted sunflower meal was much lower (49.0 mg) than that of animals given urea supplementation (88.5 mg; $n = 4$). The digestibility of UDP with pepsin and pancreatin (Table III) was slightly lower in the HSFM + U group than in the other two groups. Thus, the digestibility of original crude protein (86.2%) was also lower than that obtained for the untreated and heat-treated extracted sunflower meal.

Analysis of the crude protein fractions of the supernatant revealed that only a smaller part of RDP is degraded to ammonia and organic acids, most of it is only dissolved or degraded to peptides and free amino acids. From these results it can be assumed that not only the feed protein hitherto considered UDP reaches the abomasum and is digested there, but also a certain part of the RDP serves directly for supplying the host animal with amino acids.

During the first phase of digestion, a significant difference between SFM and HSFM was found only in the ammonia content of the supernatant. This was accompanied by a rise of the free amino acid content in the heat-treated samples (though this increase was not significant; Table IV). From combined evaluation of these two data it appears that in these samples deamination of the amino acids probably decreased.

The recovery rate of crude protein from the supernatant of HSFM + U was considerably lower than from that of the other two samples (SFM: 92.5%; HSFM: 92.2%; HSFM + U: 80.7%), a factor that makes interpretation of the results difficult. Interpolating the results to identical crude protein recovery rates (92%), only the proportion of dissolved protein (30.7%) and that of dissolved peptide (13.2%) were lower as compared to SFM.

The present studies have revealed that heat treatment reduces the rumen degradability of the crude protein of extracted sunflower meal. Urea supplementation does not affect the degradability of the heat-treated protein. During protein degradation in the rumen, only a part of the RDP fraction is degraded to organic acids and ammonia, and the other part is only partially degraded (to dissolved protein, peptides and amino acids). Having retained their amino group, the latter compounds also serve as direct amino acid sources for the host animal.

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RECENT FINDINGS AND FUTURE PERSPECTIVES OF DIGESTIVE PHYSIOLOGY IN RABBITS: A REVIEW*

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The rabbit is one of the most common laboratory animals. Consequently, there is an enormous number of publications concerning its physiology. The present review is restricted to a special field and deals with the new findings of the rabbit's digestive physiology. For the sake of better intelligibility the most important antecedents are also given.

First the particularities of feed intake regulation are demonstrated. The strong control mechanism can be disturbed by feed antinutritives, mycotoxins and high environmental temperature. Caecotrophy depends on three main factors: stimulation of rectal mechanoreceptors, perception of the specific odour of the soft faeces, and the inner motive determined by the blood level of metabolites and hormones.

The species characteristics of proportions, pH conditions, microflora and -fauna of the rabbit's digestive tract are given. The digestion and absorption of dietary nutrients are discussed. Special sections deal with the sorption of electrolytes and VFAs in the gut, the caecal digestive process, the formation of hard and soft faeces and the role of the caecotroph in stomachal carbohydrate degradation.

A relatively new area, i.e. the development and maturation of the gastrointestinal tract, is also shown. The postnatal evolution of digestive enzyme activity is also summarized. The main endogenic factors (breed, sex, age, stress, caecotrophy, presence of hairball in the stomach) influencing the digestion of dietary nutrients are also described. The probable future trends are given, too.

Keywords: Rabbit, digestive physiology, review.

The rabbit is one of the most common laboratory animals. Consequently, there is an enormous number of publications concerning its physiology. As an example, the excellent review of Nordio-Baldissera (1980) can be mentioned, which dealt with reproductive and digestive physiology, containing 60 pages and based on 426 articles. Considering the limited presentation time, the invited lecturers of the 3rd World Rabbit Congress confined their paper to one special topic: namely Gallouin (1984) spoke about the caecotrophy-related questions. The present review deals mainly with new findings in digestive physiology of rabbits, without giving full details of the previous, fundamental data. For a more detailed, methodical, up-to-date description of the theme see Cheeke (1987).

* Based on the main paper of the Physiological Section of the 4th World Rabbit Congress (Budapest, 12 October 1988)

Feed intake regulation

The rabbit's feed intake depends basically on two factors, i.e. on the feeling of hunger and appetite. The first is the result of some objective physiological functions: the decrease of the blood glucose, amino acid, lactic and volatile fatty acid level, the desiccation of buccal and pharyngeal mucous membranes, and the contractions of the empty stomach (Le Bars, 1976; Hörnicke, 1978). On the contrary, appetite is a subjective, acquired phenomenon, which contains not only the habit of the feeding time, but also the preference of the animal. The latter is especially important in rabbits, because the individual differences are great (Cheeke, 1974; Fekete and Lebas, 1983).

"Feed intake according to energy" is a characteristic feature of the rabbit. It means that, between certain limits, the daily dry matter intake is determined by the actual energy need of the animal (Lebas, 1975; Dehalle, 1981; Maertens and De Groote, 1981). This self-regulating capacity is still underdeveloped in young animals and it can be considered to be of full value approximately from the 35th day of life (Fekete and Gippert, 1985).

The regulating mechanism can be disturbed by some antinutritive substances of feeds (Champe and Maurice, 1983). Continuous consumption of a diet of subtoxic T-2 concentrations (12.5 and 25 ppm, i.e. 0.19 and 0.28 mg per kg body mass) decreased voluntary feed intake by 60 and 70% respectively (Fekete et al., 1988*a*). Among the other influencing factors one has to emphasize the environmental temperature: above the optimal 15–17 °C the feed intake decreases (Papp et al., 1983).

For the sake of logical unity the regulation of caecotrophy should be described here. It depends on three main factors, i.e. the stimulation of rectal mechanoreceptors, the perception of the specific odour of the soft faeces, and the inner motive determined by the blood level of metabolites and hormones. For the latter we have only indirect evidences. Namely, in case of an energy deficiency of the organism the rabbit consumes the total quantity of the produced caecotroph (Kalugin, 1980). During *ad libitum* feeding the dry matter intake depends on the protein requirement of the animal or, more precisely, on the protein and fibre level of its ration (Fekete and Bokori, 1985). This means that caecotrophy is higher if the feed mixture contains less protein and/or more fibre. This has been confirmed indirectly by De Blas et al. (1986) who observed a decrease in the stomachal caecotroph content on a low-fibre diet, presumably owing to a reduced intake. These findings are close to those of Roger and Leung (1973) who found in cats a depression of feed intake and avoidance of diets containing an excess of amino acids. The most commonly accepted idea is that the metabolites mediating the amino acid effect are the amino acids themselves, through some receptor system in the gastrointestinal tract, liver and/or the brain. The production of the soft faeces is practically

independent of the amount and composition of the ration (Laplace, 1978). It is the composition of the caecotroph that varies according to the diet (Proto, 1968).

The time of feed intake is a regulating factor of drinking, kinetic activity, and excretion of urine and faeces (Jilge, 1986).

Digestion and absorption. Species characteristics

The relative volume of the different sections of the digestive tract is different from that in other domestic animals. From the functional point of view it is worth mentioning that the rabbit has the largest stomach and caecum (35.5 vs. 42.8% of the total wet content respectively) among the monogastric animals (Lebas and Laplace, 1972). There is a significant difference between the static and the so-called functional volume, the latter estimated at 0.67 kPa overpressure: 53 vs. 297 ml/$W^{0.75}$ (Herd and Harrop, 1978). The pH value varies in the different parts of the digestive tract: on an average, it is 2.0–2.2 in the stomach, 6.1 in the duodenum, 7.3 in the jejunum, 7.2 in the ileum, 5.7–6.1 in the caecum and 6.1–6.5 in the colon (Matthes, 1981; Morisse et al., 1985). The dry matter content of the caecal material is 20–25%. The acidic pH of the caecal content is due to microbial fermentation which produces VFAs from fibre (Marty and Raynaud, 1966; Vernay and Raynaud, 1975).

The total germ count in the caecum is 10^9/gram content, in the colon and rectum it is 10^8 and in the jejunum it is 10^{4-5}/gram. Bacteria of the genus *Bacteroides* occur in the largest numbers (10^{2-9}/g). Owing to the feeding, lactobacilli, clostridia and *E. coli* bacteria (10^{1-3}/g) can appear (Weber et al., 1974; Matthes, 1981). Forsythe and Parker (1985*a*) reported on a rabbit-specific saccharomyces, *Cyniclomyces guttulatulus* (10^6/g) in the caecum. In the same digestive section of the healthy rabbit Lelkes and Chang (1987) found protozoa in a concentration of 10^6/ml of content.

Digestion and absorption of dietary nutrients

There are few data concerning the characteristics of the secretion and composition of saliva in rabbits. It is widely accepted that, as in other mammals, saliva is formed by a two-stage process in which an isotonic primary fluid with constant, plasma-like electrolyte composition is first formed by the secretory endpieces and then modified in the gland excurrent duct system by reabsorption of sodium and chloride and secretion of potassium and bicarbonate (Young and Van Lennep, 1979). Case et al. (1981) studied the role of extracellular anions in both phases. Neither of the major extracellular anions (Cl^- or HCO_3^-) is essential for primary fluid secretion, provided that extracellular pH is maintained at 7.4. With respect to ductal modification of the

primary saliva, HCO_3^- omission inhibits ductal Na^+ absorption (i.e. salivary sodium concentration will rise).

The degradation of proteins begins in the stomach under the influence of pepsin–hydrochloric acid complex, which is capable of hydrolysing peptide bonds of most proteins, mucin being one important exception (Tennant and Hornbuckle, 1980). The microbial protein of the caecotroph can be digested only after pretreatment of a specific bacteriolytic factor, presumably equal to colonic lysozyme which is incorporated in the soft faeces and by means of caecotrophy it is transferred to the stomach (Villard and Raynaud, 1968; Camara and Prieur, 1984). The digestion of proteins continues in the small intestine owing to the activity of pancreatic trypsin, chymotrypsin, and carboxypeptidases A and B. The intestinal mucosa contains a wide range of aminopeptidases which complete the process of protein digestion. Amino acids enter the rabbit jejunal brush burder membrane vesicle via three major routes: nonsaturable simple diffusion, Na-independent carriers, and Na-dependent carriers (Stevens et al., 1982). The relative contribution of transport pathways depends on the concentration of the amino acid in question. Six fundamental categories of transport systems have been described: simple diffusion, NBB, IMINO, PHE, y^+ and L. Transport across basolateral membranes occurs via passive diffusion and the Na-independent (L) and N-dependent (A, ASC) carriers, found universally in nonepithelial membranes (Stevens et al., 1984). The residue of dietary protein and digestive enzymes gets into the caecum, where it will be utilized by the microorganisms.

The digestion and absorption of lipids take place similarly as in other monogastric animals by means of pancreatic lipase, sterol ester hydrolase, phospholipase A, bile salts and a co-lipase (Carey et al., 1983). The most important bile acid of the rabbit is glycochenodeoxycholic acid. Ursodeoxycholic and lythocholic acids also occur but in smaller quantities. Average bile production per hour varies between 3.7 and 9.2 ml, the total acid concentration (mg/100 ml bile) ranges from 72 to 299. The bile acids are excreted conjugated mainly with glycine and in a smaller proportion with taurine (Gregg and Poley, 1966). On the contrary, Dehbi et al. (1985) found that deoxycholic acid and lythocholic acid are dominant and the amount of cholic and cheno-deoxycholic acids is negligible. The production shows a circadian rhythm. This contradiction probably derives from the different experimental techniques. Approximately 70% of the rabbit's bile pigments is biliverdin because, due to the low biliverdin reductase activity of the liver, the synthesis of bilirubin is limited (Munoz et al., 1986).

The readily available carbohydrates (sugars, starch) can be digested completely and their absorbed end products are glucose, fructose and galactose. It is worth mentioning that the up-to-date model of transepithelial glucose transport is based on experiments using rabbit's jejunal brush-border and rat

basolateral membranes. It was stated that glucose crosses the intestinal brush-burder via one diffusive and two saturable systems (Kaunitz and Wright, 1983). The independent glucose carrier of enterocyte basolateral membrane resembles the facilitated glucose carrier of other membranes. The hydrolysis of fibre components (pectin, hemicellulose, cellulose) is accomplished by the intestinal bacteria, mostly those of the caecum. The end products of that fermentation are short-chain fatty acids. The proportion of VFAs in the digestive tract reflects this process: small intestine (total): 4.8, ileum: 11.1, caecum: 15.4, colon: 11.7 meq/100 gram content (Marty and Carles, 1973; Marty et al., 1973). VFA concentration (especially that of butyric acid) falls after starvation.

The produced VFAs (formic, acetic, propionic, butyric) and the other organic acids, remaining from the feed (malonic, fumaric, succinic acid) are actively absorbed through the caecal and colonic wall into the blood. The intestinal wall utilizes a part of these acids (mainly butyrate) as a source of energy, so the end product of this process, lactic acid, will get into the blood too (Beauville et al., 1974). The proportion of the three main short-chain fatty acids, acetic, propionic and butyric acid in the caecum is 10 : 1 : 1.5–3 (Morisse et al., 1985).

Heat and chemical treatment influences the digestibility of starch. Blood glucose and insulin concentration does not increase in rabbits fed with crude starch. Heat treatment (100 °C) enhances the hydrolysis. The quickest hydrolysis and absorption can be obtained by feeding acetylated distarch adipate (Lee et al., 1985).

Sorption of electrolytes and VFAs in the gut

The duodenal mucosa possesses a considerable capacity for bicarbonate secretion. The absorption of HCO_3^- by the jejunum is an active, metabolically driven process. The rabbit ileum has a considerable capacity for alcalization (bicarbonate secretion) of chyme. There is an interrelationship between bicarbonate secretion and NaCl absorption (Hopfer and Liedtke, 1987). Na absorption and Cl absorption in the ileum are linked and can be explained by Na $\leftrightarrow$ H and Cl $\leftrightarrow$ HCO_3 exchanges (Nellans et al., 1974). During the excretion of hard faeces, chloride will be actively absorbed from the proximal colon and secreted in the distal colon. Its absorption from the caecum is passive, i.e. the chloride concentration of the caecal content has to exceed that of the blood plasma (95 meq/l). There is no K movement in any direction in the caecum (Leng, 1978). The caecal appendix secretes an alcaline fluid of high bicarbonate concentration (William et al., 1961). From the caecum there is a rather passive absorption of bicarbonate: the threshold is 19 meq/l. The transport of chloride and that of bicarbonate are in connection. The proximal colon actively absorbs water, sodium and chloride, and secretes bicarbonate and potassium, whereas

the distal colon absorbs sodium and water and secretes potassium and chloride (Clauss, 1978, 1985). The bicarbonate secretion of the colon moderates the pH fall owing to the rising VFA level (Garcia et al., 1982; Vernay et al., 1984; Vernay, 1986).

Intracellular Ca (with calmodulin) is involved in the regulation of NaCl absorption in rabbit ileum and K secretion in the descending colon. Most of the effect of Ca on intestinal transport is mediated by initiation of arachidonic acid metabolism (PGE_2). Increase in intestinal cAMP also alters active Na, Cl and K transport. Agents that increase intestinal cAMP content (e.g. bacterial toxins, bile salts etc.) will inhibit absorption of sodium and chloride and increase K secretion (Donowitz and Welsh, 1986).

Digestive processes in the caecum

The concentration of the bacterial flora in the caecum is 10^{9-11}/gram, with the predominance of the strictly anaerobic *Bacterioides*. The main N source for the bacteria is NH_3 produced by their dezaminase and urease activity. Cellulolytic acitivity is low, so the energy required for the assimilation of ammonia will be obtained from the degradation of sugars, pectin and xilase. In case of an incomplete protein supply the blood urea will be excreted in the caecum. A certain amount of urea derives from the feed (Knutson et al., 1977). The biuret can be a more efficient N source than urea (Proto and Gioffre, 1986). Urea will be hydrolysed mainly by bacteria adhering to the mucous membrane: *Clostridium coccoides*, *Cl. innocuum*, *Peptostreptococcus productus*, *P. micrus*, *Peptococcus magnus*, *Fusobacterium russii* (Forsythe and Parker, 1985*a*).

As a function of the protein level of the feed and the NH_3 concentration of the caecal content, the produced ammonia will be built into the bacteria and will be utilized by caecotrophy (Robinson et al., 1986). The described utilization of urea is hindered by feeding antimicrobial substances (Houpt, 1963). Besides the protein concentration of the feed mixtures required for production, the NH_3-assimilating capacity of the caecal flora is trifling, so supplementation of the rabbit's feed with urea does not result in practical benefit. Namely, the average ammonia concentration of the caecum is 5 mmol/l, while the effective NH_3-assimilation occurs at about 1 mmol/l (Forsythe and Parker, 1985*b*). It is worth mentioning that a certain proportion of ammonia produced in the mucous membrane gets into the blood again.

Caecotrophy

Taxonomically distant mammals (some rodent species, beaver, herbivorous monkeys etc.) developed a particular mechanism allowing the separate excretion of the caecal content rich in nutrients and the normal, hard faeces

poor in valuable material. The reingestion of caecal content (caecotroph) makes possible the better utilization of nutrients. The caecotroph will be swallowed without chewing. The typical composition of the two types of faeces is the following: dry matter 52.7 vs. 38.6, crude protein 15.4 vs. 34.0, ether extract 3.0 vs. 5.3, crude fibre 30.0 vs. 17.8, ash 13.7 vs. 15.2, N-free extract 37.9 vs. 36.7%, gross energy 18.2 vs. 19.0 MJ for hard faeces and caecotroph, respectively (Fekete and Bokori, 1985). The caecotroph and the hard faeces will be produced in the proximal colon, at different times. During the production of hard faeces a separating mechanism, by the concentrations of the colonic haustrae, removes the fluid and small particles in the caecum. The process is helped by the active water secretion of the colon (Ehrlein et al., 1983). The fusus coli is supposed to be the pacemaker of the contractions (Ruckebusch and Fioramonti, 1976). The described separating mechanism does not act during the excretion of the caecotroph, which is fundamentally a caecal content, enclosed by mucin produced by the colonic cells (Bonnafous and Raynaud, 1967; Björnhag, 1972; Gallouin and Demaux, 1980). The mucilaginous membrane inhibits the diffusion of ions and absorption of electrolytes.

During the formation of hard faeces the caecal content will thoroughly be transformed in the colon. The wall of the proximal colon produces a lytic factor which dissolves the bacteria, especially those of low carbohydrate content (Bonnafous and Raynaud, 1978). The distal colon produces, mainly in the soft faeces phase, a species-specific lysozyme, which will get into the stomach with the caecotroph and there it has a bacteriolytic role (Camara and Prieur, 1984).

The sodium, water and chloride absorption of some parts of the proximal, and the total distal colon increases during the hard faeces phase (Hörnicke et al., 1984). It is aldosterone secretion which is responsible for the enhancement of these transports (Vernay, 1985), but Na^+, Cl^-, HCO_3^- and water absorption in the caecum, as well as Cl^- secretion in the distal colon, are independent of the blood glucocorticoid level (Schäfer, 1985).

Clauss and Hörnicke (1979) kept rabbits in regulated light circumstances (12 dark and 12 light hours). Caecotrophy fell between 8 and 12 hours. Aldosterone concentration of the blood plasma and the electric potential difference showed a diurnal rhythm. The Na to K ratio of excretum varied between 0.2–1.2 and reached its maximum in the soft faeces phase. Blood aldosterone concentration is the lowest in the caecotroph phase, consequently the sodium level and the Na to K ratio of the caecotroph will be higher than in the hard faeces (Clauss, 1985). According to Fekete et al. (1988) the Na to K ratio of the caecotroph is 0.6 and that of the hard faeces is 0.4. In absolute value the caecotroph contained more from both elements. ACTH treatment also had an aldosterone-like effect on the hard faeces sodium to potassium ratio, which shifted to 0.3. This is close to the result of Dürr and Clauss (1984) who des-

cribed stimulation of the Na^+-permeability of the descending colon by dexamethasone or aldosterone.

The role of caecotroph in stomachal carbohydrate degradation

The starch of the feed will be hydrolysed under the action of amylase into glucose molecules. Amylase can derive from three sources: feed, saliva and caecotroph bacteria. The third is the most important since it is less sensitive to the acidic milieu. The produced glucose diffuses into the caecotroph balls and will be transformed into lactic acid and carbon dioxide (Griffiths and Davies, 1963). Lactic acid rediffuses into the lumen: its absorption begins in the stomach but is completed only in the small intestine (Hörnicke and Mackiewicz, 1976). The mucous membrane, surrounding caecotroph pellets, plays an important role: it protects the bacteria from the influence of gastric juices (Hörnicke, 1986). In my opinion, the importance of the described phenomenon is not the energy supply by lactic acid, but rather the lightening of the hind but's carbohydrate overload, as pancreatic amylase production of the rabbit is relatively modest (Catala and Bonnafous, 1979), and the acidification of the stomachal content and activation of pepsinogen.

Development and maturation of the gastrointestinal tract

The rabbit's digestive tract reaches its adult proportions in the 4th month of life (Lebas and Laplace, 1972). Marked changes occur in villous membrane composition, epithelial cell differentiation and transport processes during this period. The maturation of the small intestine functional unit (i.e. the crypt-villus structure) is very important in the digestive processes. The phospholipid and cholesterol concentration and specific fatty acid content of the microvillous membrane of the small intestine change with age (Pang et al., 1983: Schwarz et al., 1984). The results obtained on rats suggest that there are essential developmental changes in the glycoprotein content and coat of the intestinal mucous membrane, too (Sheard and Walker, 1988). These differences affect membrane fluidity, organization and receptor function. The mucosal barrier function is also immature in the newborn rabbit: intestinal antigen intake and endotoxin binding are greater than in adult animals (Udall et al., 1981; Bresson et al., 1984). The feed antigens are absorbed more readily into the circulation during the first weeks of life. According to Lecce and Broughton (1973), this period lasts up to the 23rd day of life. In the opinion of Štěpánkova et al. (1983) only the first 8 hours are important in this respect.

The described processes are not independent of the feeding. The small intestine of rabbits fed with doe's colostrum will be greater, heavier, and contain more DNA than that of young rabbits fed artificially during the first day after birth (Hall and Widdowson, 1979). These results suggest the presence

of a specific trophic and/or stimulating effect on the development of the gastrointestinal tract. There is some evidence of the potential role of colostral epidermal growth factor (EGF), nerve growth factor (NGF), somatomedin-C, insulin-like growth factor, insulin, corticosteroids, thyroxine, taurin, glutamine and amino sugars (Sheard and Walker, 1988). Gastrointestinal hormones (primarily enteroglucagon, gastrin and motilin) induced by feeding may also modify the whole process. The formation of the normal enteral microflora is a very important factor in the development of ileal villi (Boot et al., 1985).

Development of digestive enzyme activity

The activity of digestive enzymes of the suckling period, except lactase, is low in the first two weeks (Deren, 1971). In the third week, parallel with the decrease of lactase activity, the production of saccharase, maltase, trypsin, chymotrypsin and amylase increases (Lebas et al., 1971; Henschel, 1973; Alus and Edwards, 1976). As to the proteolytic enzymes, this is a so-called two-step development of enzyme activity (Corring et al., 1982). Bile acid production undergoes a threefold increase during the first 20 days (Croizat and Lambiotte, 1971).

The above-mentioned change is not caused by weaning (i.e. the change of feeding), but by the hormonal status of the organism. Corring et al. (1972) found that prolongation of the suckling period did not influence either the time of intestinal closure or the development of pancreatic amylase, lipase and chymotrypsin activity, although the lactase activity could be maintained. The fibre-starch ratio of the postweaning feed had no influence on amylase excretion by urine (Fekete et al., 1988*b*). On the basis of rat experiments Daniels et al. (1972), Chan et al. (1973) and Henning (1985) explain the cessation of intestinal macromolecule uptake by the elevation of blood cortisol and thyroxine. Exogeneous glucocorticoid and ACTH injections stimulate the production of pepsinogen and pancreatic enzymes (Courtot, 1972) as well as that of sucrase, maltase, trehalase, amino peptidase (Galand and Forstner, 1974) and alcaline phosphatase (Moog, 1953). Administration of T_4 or T_3 has also been shown to cause a precocious decline of jejunal lactase activity and ileal lysosomal hydrolases (Paul and Flatz, 1983; Koldovsky and Herbst, 1971) and precocious increases in jejunal sucrase and maltase activity (Jumawan and Koldowsky, 1978).

Although some of the data derive from rat and mouse experiments, one can state that the development of the digestive enzyme set depends upon the function of the hypophyseal–adrenal axis. The compounds of the feed (as substrates) contribute to the hormonal stimulus, stabilizing the production of the enzymes ("enzyme, stabilized by its substrate").

Main endogenic factors influencing digestion, evaluated by digestibility trials

The digestibility of nutrients is slightly lower in the Californian than in the New Zealand White rabbit (Hullár and Gippert, 1986). No sex differences were found in respect of digestion either in the growing or in the adult rabbit. The digestion coefficient of protein is by 3.16% higher in the growing rabbit than in the adults (Fekete and Lebas, 1983; Fekete and Bokori, 1986*a*).

The stimulated "physiologic" stress (0.5 IU ACTH/kg body mass/day im.) did not influence the digestibility of nutrients, either with or without antibiotic supplementation. At the same time sodium to potassium ratio of the faeces changed (Fekete et al., 1988).

Ad libitum fed rabbits consume their soft faeces (caecotroph) according to the "protein minus fibre" level of the ration. This means that the relative protein deficiency or fibre surplus enhance, while the inverse situation diminishes caecotrophy and may modify the measured digestibility coefficient (Fekete and Bokori, 1985).

The presence of hairball in the stomach reduced protein and fibre digestibility and resulted in a negative apparent absorption coefficient of ash, with the simultaneous elevation of the blood glucocorticoid level (Fekete and Bokori, 1986*b*).

Future trends

The last ten or fifteen years have brought unexpected progress in research into the digestive physiology of rabbits. Still, great challenges have remained. In years to come, research will probably concentrate on some of the following topics.

- Particularities of the regulation of feed and caecotroph intake.
- The process and control of maturation of the gastro-intestinal tract.
- Developmental aspects of digestion: enzymatic and transport development, formation of microbial digestion in the large intestine.
- Details of digestion and absorption of nutrients. The influence of physiological status (e.g. sexual cycle), presence of gastrointestinal parasites etc.
- Further investigation of the morphology and biochemistry of the rabbit's intestinal flora, its role in the digestion, possible biotechnological alterations.
- Control and integration of gastrointestinal functions with special emphasis on gastrointestinal hormones and on neural and hormonal control of intestinal ion transport.

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Acta Veterinaria Hungarica 37 (3), pp. 281–288 (1989)

DEVELOPMENT OF PORCINE DIGESTIVE ENZYMES WITH SPECIAL REFERENCE TO RIBONUCLEASE

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The activity of digestive enzymes was determined in the gastrointestinal tract of newborn, 1-, 3- and 5-week-old piglets and in adult pigs. The pancreas of newborn piglets contained considerable ribonuclease (RNase) activity, which continued to increase with age. After the initial values an opposite tendency was found in the intestinal contents, probably due to the increase of proteolytic degradation with advancing age. Serum RNase showed little age dependence. The time-course of development of pancreatic RNase resembled more that of proteolytic enzymes than that of amylase.

The data indicate that a high pancreatic secretion of RNase commences much before the appearance of RNA in the diet.

Keywords: Digestive enzymes, ribonuclease, age dependence, pig.

Pancreatic ribonuclease (RNase) catalyzes the first step in the degradation of dietary RNA, allowing the utilization of nucleotide components: phosphate, ribose, pyrimidines and purines. RNase also constitutes a first-line defence against foreign genetic information. Although numerous workers studied the occurrence and molecular properties of pancreatic RNase, we are not aware of the occurrence and function of RNase in the suckling age, when the usual diet, milk, is free of nucleic acid. Therefore, we determined RNase activity in the pancreas, intestinal contents and serum of piglets of different age. For comparison, the activity of several other digestive enzymes was measured, too.

Materials and methods

Seventeen Large White × Landrace piglets of different age and from different litters were sacrificed. The newborn, 1-week-old and 3-week-old piglets were suckled by the sow, but the 5-week-old piglets had been weaned to prestarter *(ad libitum)* 5 to 7 days before killing and were kept in cages in the last days. The age of piglets in this group was determined with an error of ±2 days and for the rest of piglets with ±half day. The youngest group of

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piglets was born at night, sucked colostrum normally and was killed in the morning. A group of adult pigs with about 110 kg live mass was slaughtered after approximately 6 h of starvation.

Blood, pancreatic tissue and gastrointestinal contents were taken from the animals at slaughter. Blood was allowed to clot and serum was removed. Samples were refrigerated until use.

From the pancreas, fat and connective tissue were removed and the rest was ground in a mortar, together with a few drops of 0.05 M $CaCl_2$ solution, then filled up with the same solution to a 1 : 100 final dilution. Intestinal and gastric contents were weighed and diluted 1 : 100 with saline.

Before determining pancreatic proteolytic activities, zymogens were activated with porcine duodenal extract containing enterokinase. Five μl of duodenal extract was added to 5 ml pancreatic extract and filled up to 7.5 ml with 0.1 M, pH 8.0 Tris-HCl buffer. The mixture was incubated at 40 °C for 15 min in a water bath.

The duodenal extract was prepared as follows: 10 cm duodenum of an adult pig was slit open and carefully rinsed in saline. Then the mucosa was disintegrated by agitating the tissue with a glass rod in a tenfold volume of saline. After centrifugation at 8000 *g* for 10 min, the clear supernate was used for activation.

Ribonuclease was determined according to McDonald (1955) in 0.1 M, pH 5.0 acetate buffer at 40 °C. Undigested substrate was precipitated by McFadyen's reagent and removed by centrifugation. Digested RNA was read at 260 nm in the supernate. Yeast RNA substrate (Merck) was purified by precipitation in ethanol, before use. When determining serum RNase, RNA--free serum blanks were incubated parallel with the experimental tubes. Incubation of blank was found superfluous for pancreatic and intestinal samples.

Alpha-amylase was determined by the iodine-starch method of Somogyi (1938) and gastric proteolytic activity (pepsin) with denatured haemoglobin in 0.06 M HCl, according to Anson (1938). Pancreatic and intestinal proteolytic activities were measured in 0.1 M, pH 8 Tris-HCl buffer. Azocasein substrate was prepared according to Bergmeyer (1970) and used for the determination of total alkaline proteolytic activity. Trypsin and chymotrypsin were determined by direct spectrophotometric reading with benzoyl-L-arginine ethyl ester (BAEE) and benzoyl-L-tyrosine ethyl ester (BTEE) substrates, respectively (Schwert and Takenaka, 1955). The values were corrected by 3.75%/°C, when temperature differed from 25 °C. Rennet activity was detected qualitatively by clotting of commercial cow's milk (Soxhlet, 1877).

Activities were calculated to g wet tissue or wet gastrointestinal content or to ml of serum. Amylase activity was expressed in Somogyi units, ribonuclease, pepsin and total alkaline protease activities in spectrophotometer units (change of one unit of optical density per min). Tryptic and chymotryptic

activities were calculated to enzyme μg by dividing spectrophotometric units by 12 and 24, respectively.

Mean and standard deviation were calculated from the data.

Results and discussion

Sampling of pancreas required killing of animals and this fact limited the size of the experimental groups, but still allowed the detection of tendencies in enzyme development. Only the adult pigs were starved before slaughter and, therefore, pepsin values of this group are not really comparable with those of the other groups.

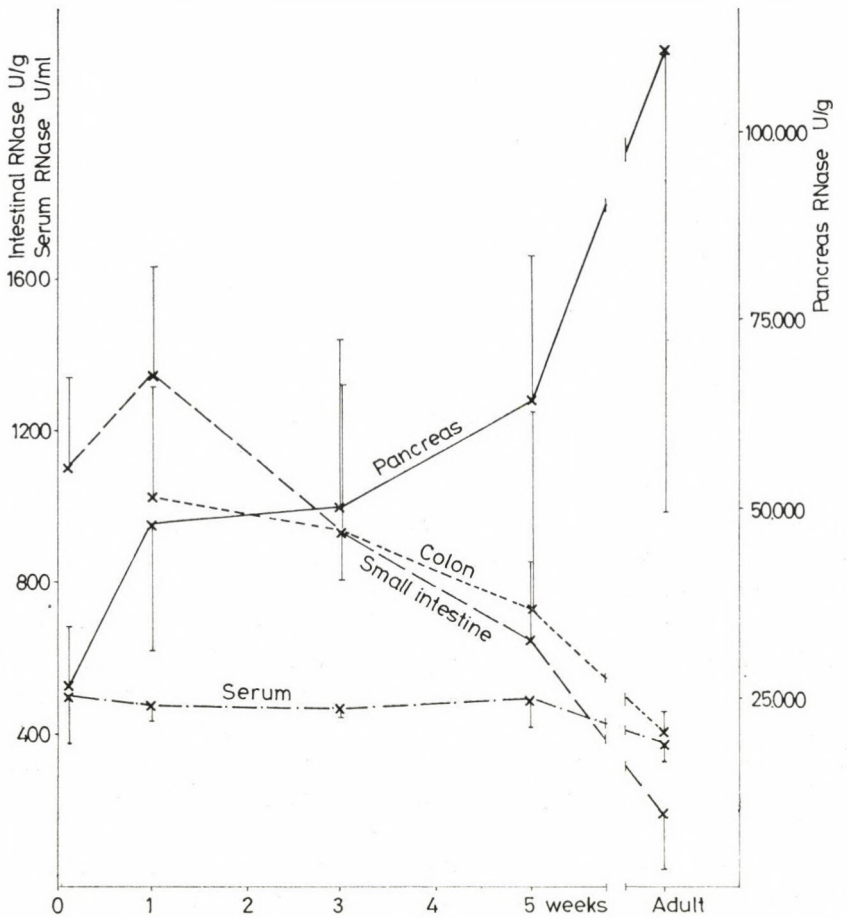

Fig. 1. Ribonuclease activity of pigs (± standard deviation). Number of animals in the groups (as in the following figures): half day: 4, 1 week: 5; 3 weeks: 4; 5 weeks: 4; adult: 8

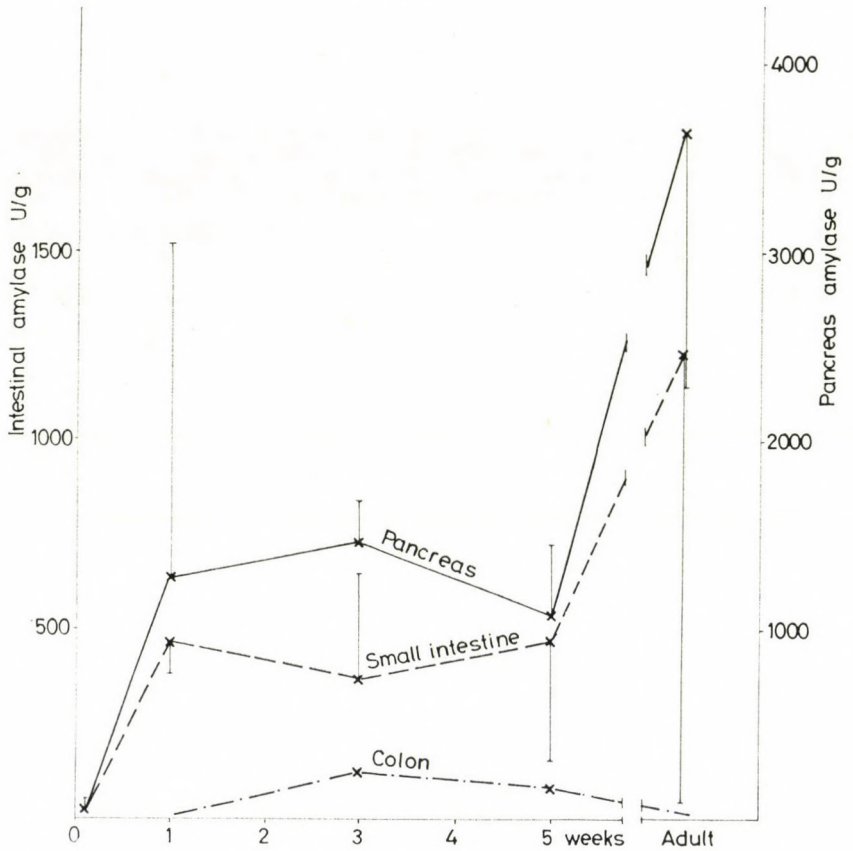

Fig. 2. Amylase activity

Despite that sow's milk does not contain RNA, the pancreas of the newborn piglet showed considerable RNase activity, which continued to increase with advancing age (Fig. 1). Intestinal RNase values did not reflect those of the pancreas and opposite tendencies were found in the two organs after the initial values. One of the possible explanations for the decreasing intestinal RNase activities is the increase of intestinal proteases at least from 3 weeks of age (Fig. 3) resulting in faster proteolytic degradation of RNase. Serum RNase activities did not change much during the suckling period, but the adult values were non-significantly lower (Fig. 1). So the present study does not provide a clue to the origin of serum RNase.

In agreement with literary data (Walker, 1959*a*; Corring and Aumaitre, 1971), *pancreatic amylase* activities showed a clear tendency to increase during development, beginning with very low levels in the newborn (Fig. 2). A similar increase was found for small intestinal amylase by Walker (1959*b*), while not

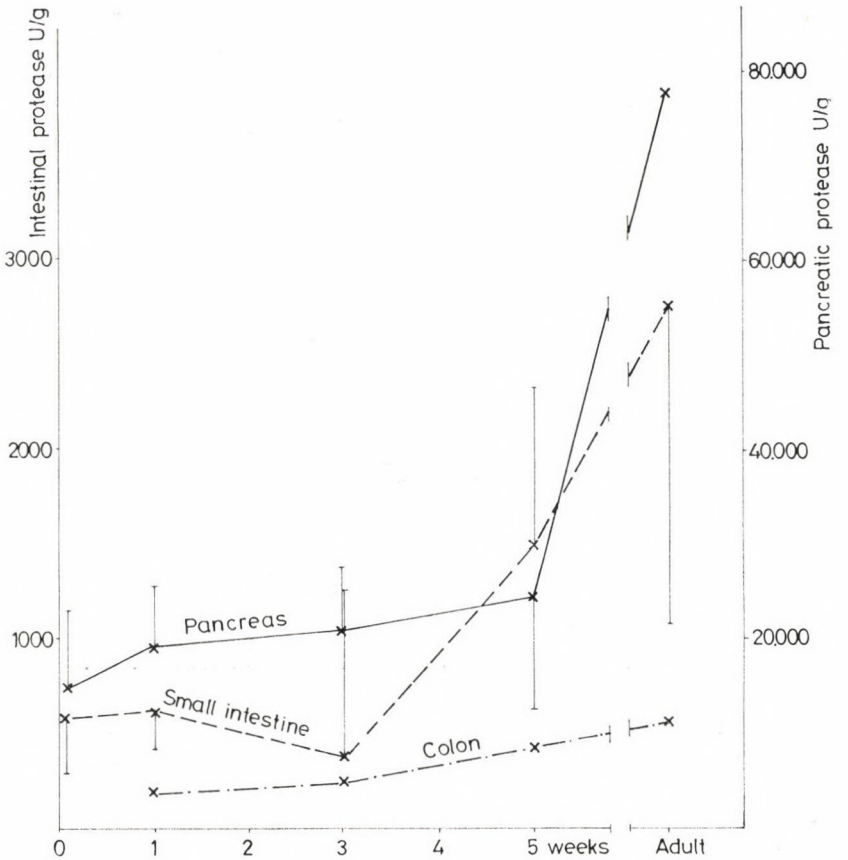

Fig. 3. Total proteolytic activity

much change was demonstrated during the suckling age in the present study (Fig. 2).

Proteolytic activities (Figs 3–5) also tended to increase both in pancreas and small intestine. Chymotrypsin clearly fell back after weaning (5-week-old group). Contrary to the findings of Corring and Aumaitre (1971), the ratio of trypsin to chymotrypsin markedly increased during development both in pancreas and small intestine. The time-course of pancreatic RNase resembled that of pancreatic proteolytic activities more than the changes of pancreatic amylase. The failure to detect trypsin in the intestinal content of newborn piglets is due partly to ingestion of inhibitor with colostrum, as it was shown earlier (Baintner, 1973).

In agreement with the findings of Lewis et al. (1957), acid proteolytic activity (pepsin) tended to increase with age in the *gastric content* (Fig. 6). The exceedingly high peptic activity in the adult stomach might be due both to age difference and to the fact that the animals were at the end of the digest-

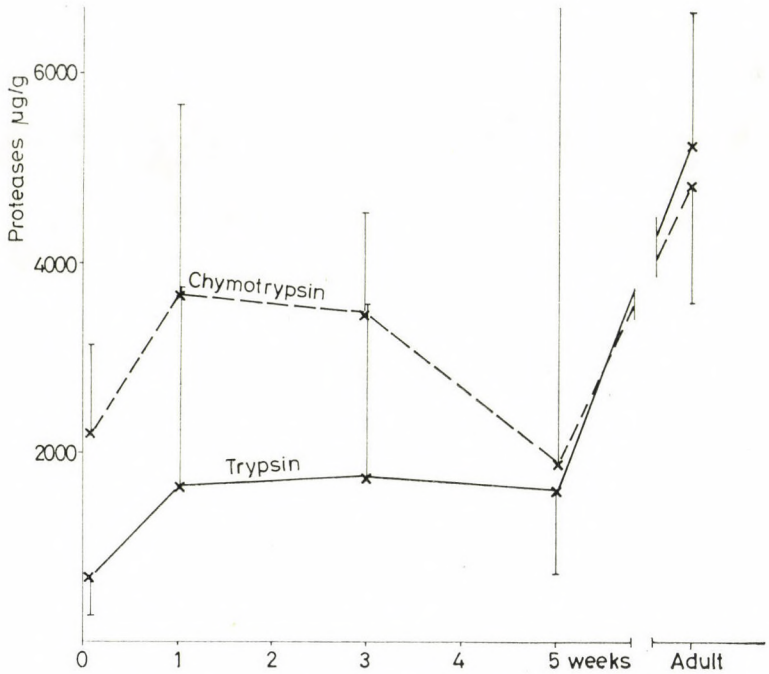

Fig. 4. Pancreatic trypsin and chymotrypsin activity

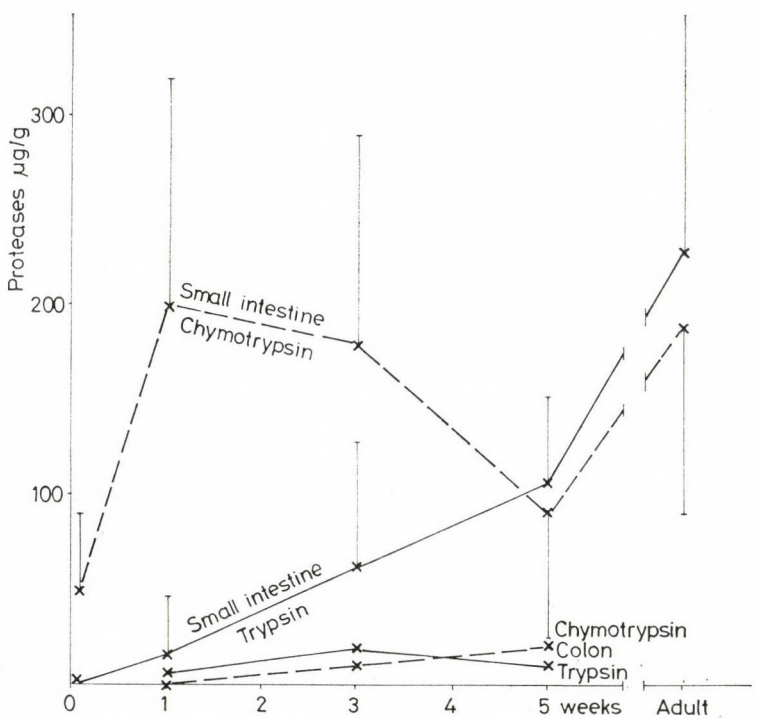

Fig. 5. Intestinal trypsin and chymotrypsin activity

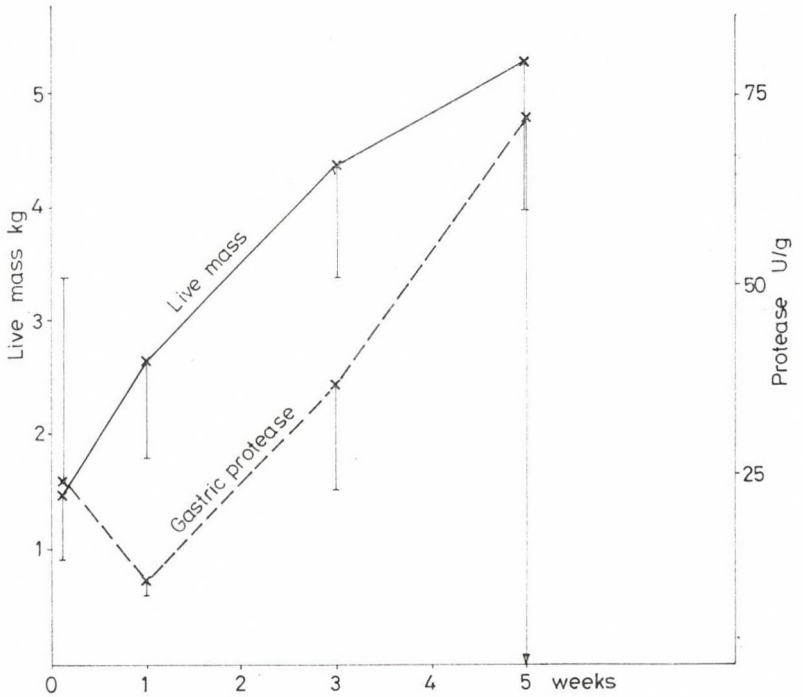

Fig. 6. Live mass and gastric proteolytic activity

ive phase after ingestion of meal, i.e. at the commencement of starvation. Rennet activity could be detected in all newborn piglets and in one of the 1-week-old animals, but it was lacking thereafter.

In the newborn piglets the *colon* still contained meconium, which was not sampled. In the older piglets the small intestinal enzymes usually much surpassed the colonic ones (Figs 1–3 and Fig. 5), with the exception of the high colonic RNase activity. Bacterial enzymes might contribute to RNase, amylase and azocaseinase activities measured in the colon content, and to the degradation of trypsin and chymotrypsin of pancreatic origin. These latter activities showed maximum in the colon at 3 and 5 weeks, respectively.

Acknowledgement

We would like to thank Miss E. Törsök for assistance.

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DEVELOPMENT OF OVINE DIGESTIVE ENZYMES WITH SPECIAL REFERENCE TO RIBONUCLEASE

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Twenty Merino lambs of four age groups (1 day, 2, 4 and 7 weeks) and 8 adult Merino wethers were killed. The development of pancreatic and gastrointestinal enzymes was followed by determining RNase, amylase, lipase, trypsin, chymotrypsin and total proteolytic (azocaseinase) activity. Pancreatic protein content, rumen and abomasal pH and abomasal clotting time were also determined.

Pancreatic RNase was already present in the newborn lambs and significantly rose in the first 2 weeks of life and before reaching adult values. The increase was more marked and went to higher adult values than in the pig (Baintner and Farkas, 1989). The time-course resembled that of pancreatic amylase and chymotrypsin; pancreatic trypsin and azocaseinase also showed some similarities, but pancreatic lipase had a different time course. Small intestinal RNase also changed differently; it showed a maximum at 4 weeks and had trends opposite to total proteolytic activity, indicating partial digestion of the enzyme by intestinal proteases. Rumen and caecal RNase activities may be indicative of microbial growth and fermentation rate; they showed mostly opposite tendencies in the two localities.

In contrast to the pig (Baintner and Farkas, 1989), pancreatic and small intestinal trypsin:chymotrypsin ratios did not show significant increase during development in sheep.

Keywords: Digestive enzyme, ribonuclease, age dependence, development, sheep.

Nutrients undergo microbial fermentation in the forestomachs of adult ruminants. At the same time, some nutrients are incorporated into, or synthesized by, the growing microbial population. The rumen liquor, containing suspended microbes and feed particles, passes to more distal parts of the gastrointestinal tract for digestion. This suspension contains much less starch than a usual ration, little lipid, but much microbial protein and nucleic acid, the latter being mostly bacterial RNA. Ruminants have adapted evolutionarily to the digestion of fermented feed: the pancreatic juice of adult ruminants has low amylase (Walker, 1959*a*) and lipase activities, but much RNase (Barnard, 1969) and zymogens of proteases (Walker, 1959*b*; Huber et al., 1961). Nucleic acid degrading enzymes save dietary and salivary (recycling) phosphates from being excreted in the faeces. Other components of nucleic acids are also reused: absorbed ribose supports gluconeogenesis, while nucleotide bases are reused via the "salvage pathway" in the host.

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In previous experiments RNase activity was demonstrated in the pancreas and intestinal contents of the pig (Baintner and Farkas, 1989) and rat (Baintner, unpublished results), already in the newborn animals. In the present work the development of pancreatic RNase was examined in growing lambs. For comparison, several other digestive enzymes were also determined in the pancreas, rumen, abomasum and intestines.

Materials and methods

Twenty Merino lambs of both sexes and of different age, obtained from a cooperative farm, were used for the study. Most of the lambs were born at night and were killed either next day or two, four or seven weeks later. Each experimental group consisted of 5 lambs. Eight adult Merino sheep were also slaughtered. They came from another flock and were 1.5 to 3 year old wethers.

Lambing began in late autumn and the last sheep were slaughtered in February. The ewes were kept in an unheated, traditional sheep stall on straw litter and were fed high-quality alfalfa hay and concentrate. The lambs suckled the ewes and had free access to both feed and water. Medium-quality grass or alfalfa hay and concentrate were fed to the adult wethers.

Both lambs and adult wethers were bled at slaughter. Twins occurred only once; one of them was killed as newborn, so there was no competition for milk. Samples were taken from the pancreas and from the contents of different portions of the gastrointestinal tract. Middle jejunal content was sampled in the wethers, but in the lambs the whole small intestinal content was pressed out and mixed. The samples were kept frozen until analysed.

Preparation of samples

Rumen liquor was prepared by pressing the liquid phase of the rumen contents through a cotton layer by hand. Abomasal and intestinal contents were weighed and diluted with distilled water. The pancreas was cleaned of fat and homogenized in ice-cold saline in a Potter-Elvehjem homogenizer. The protein content of the homogenate was determined by the method of Lowry et al. (1951) as modified by Hert (1971), using bovine albumin as reference standard.

Pancreatic homogenate was activated as follows: 0.3 ml homogenate (20-fold dilution with saline); 0.2 ml enterokinase solution (2 mg/ml in 0.15 M NaCl, Sigma); 1.0 ml 0.05 M, pH 5.6 citrate buffer.
The mixture was incubated at 37 °C for 60 min in a water bath.

Determination of enzyme activities

Ribonuclease was determined with yeast RNA (EGA-Chemie) as substrate (McDonald, 1955). Small molecular mass RNA debris was eliminated by ethanol precipitation of the high molecular mass fraction. Determination of *amylase* was performed with the Phadebas Amylase Test Kit (Pharmacia). *Lipase* was measured with oleic acid beta-naphthyl ester as substrate (Schőn et al., 1961). *Tryptic* and *chymotryptic* activities were followed by direct spectrophotometric reading, with benzoyl-L-arginine ethyl ester (BAEE) and benzoyl-L-tyrosine ethyl ester (BTEE) substrates, respectively (Schwert and Takenaka, 1955). The values were corrected by 3%, if the temperature differed 1 °C from 25 °C. Total proteolytic activity ("azocaseinase") was measured in 0.1 M, pH 8.0 tris-HCl buffer in the case of pancreatic homogenate and intestinal contents and in 0.1 M, pH 6.5 phosphate buffer in the case of rumen content (Baintner, 1981). Azocasein substrate was prepared according to Bergmeyer (1970). Clotting time, the reciprocal of which is indicative of *rennet* activity, was measured by the clotting of 1 ml commercial cow's milk by 5 μl abomasal juice at 40 °C.

Trypsin and chymotrypsin were expressed in enzyme μg, lipase, azocaseinase and ribonuclease activities in transformed substrate per min and amylase activity in nkatal. Activities were related to 1 *mg* of pancreatic protein, to 1 *ml* of rumen liquor or to 1 *g* of gastrointestinal contents.

Other methods

An OP-211 Radelkis (Budapest) pH meter was used for the determination of pH and a Spektromon 195 (MOM, Budapest) spectrophotometer for photometry.

Mean and standard deviation were calculated from the data. Group means were compared by Student's *t* test.

Results

Developmental changes of rumen pH, RNase and azocaseinase are shown in Fig. 1, live mass, abomasal pH and clotting time in Fig. 2, pancreatic protein, RNase and lipase in Fig. 3, pancreatic amylase, trypsin, chymotrypsin and azocaseinase in Fig. 4, intestinal amylase and RNase in Fig. 5 and intestinal trypsin, chymotrypsin and azocaseinase in Fig. 6.

Trypsin and chymotrypsin could not be detected in the *caecal* contents of 1-day-old and 2-week-old lambs and all caecal samples were free of these activities thereafter.

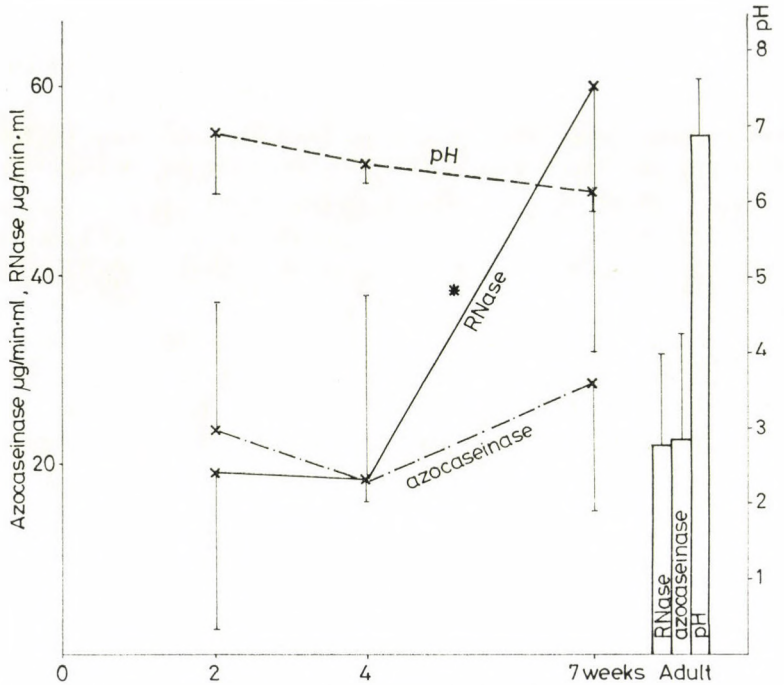

Fig. 1. Rumen RNase, azocaseinase and pH ± standard deviation (✳: significant difference at $P < 0.05$)

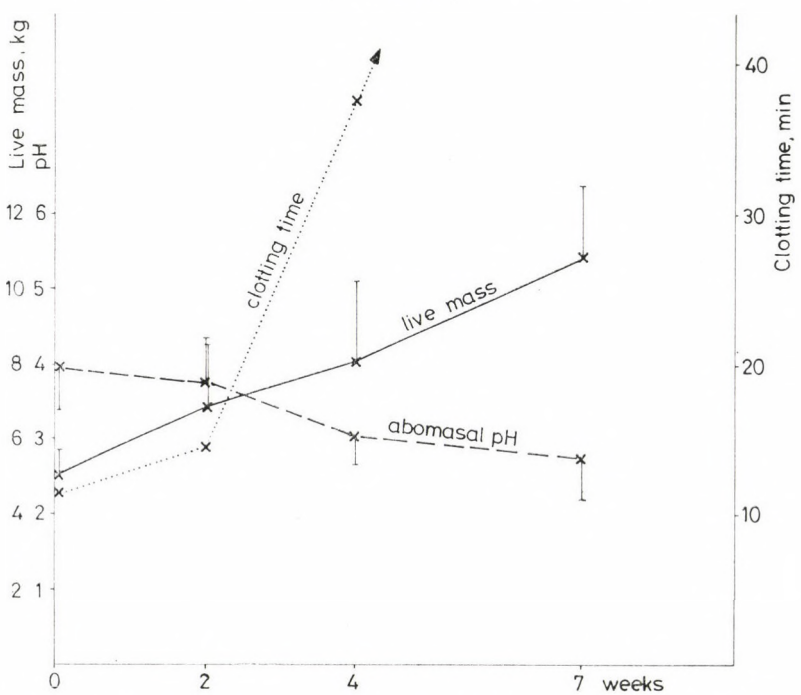

Fig. 2. Live mass, abomasal pH and clotting time ± standard deviation

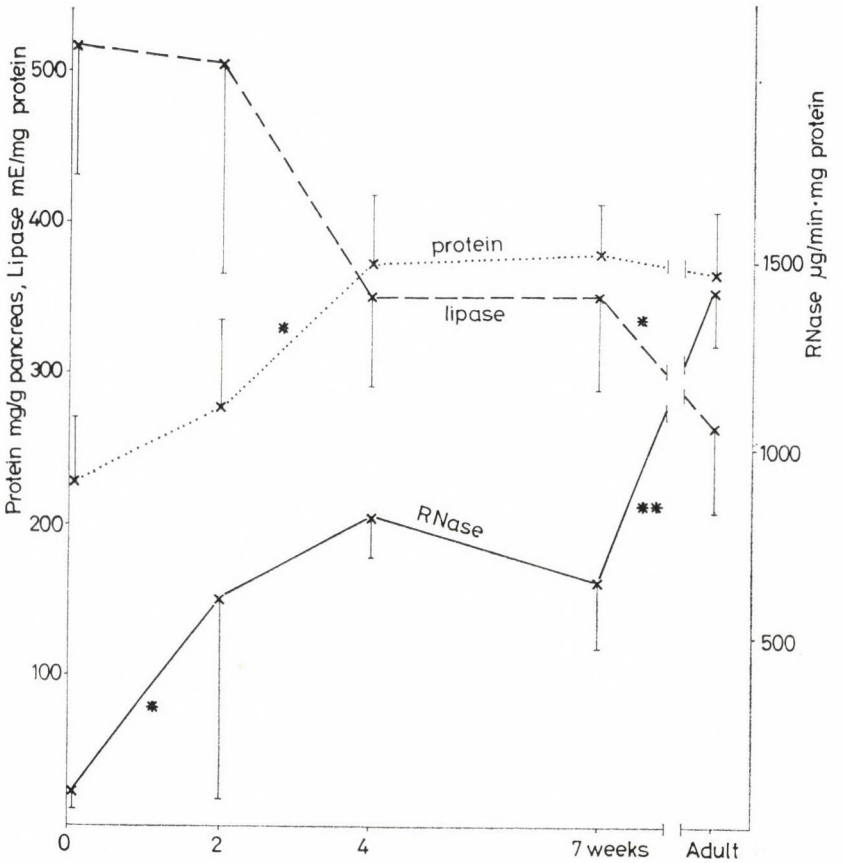

Fig. 3. RNase and lipase activities and protein concentration in the pancreas ± standard deviation. Significant differences: $*: P < 0.05$; $**: P < 0.01$

Discussion

Both ewes and lambs were healthy during the whole experimental period. Stillbirths did not occur. All *newborn* lambs (designated as one-day-old) had already suckled colostrum before killed. The rumen, small intestine and caecum contained chyme of mostly colostral origin, but the colon still held brown meconium (discarded). In this age group, the rumen contents of three out of five lambs clotted the milk added to the samples *in vitro*, indicating a reflux of abomasal content during the kill. Therefore, the rumen contents of newborn animals were not used for enzyme determinations.

In previous experiments two sorts of *protease inhibitor* were found in the alimentary tract of the newborn lamb: colostral trypsin inhibitor (Baintner,

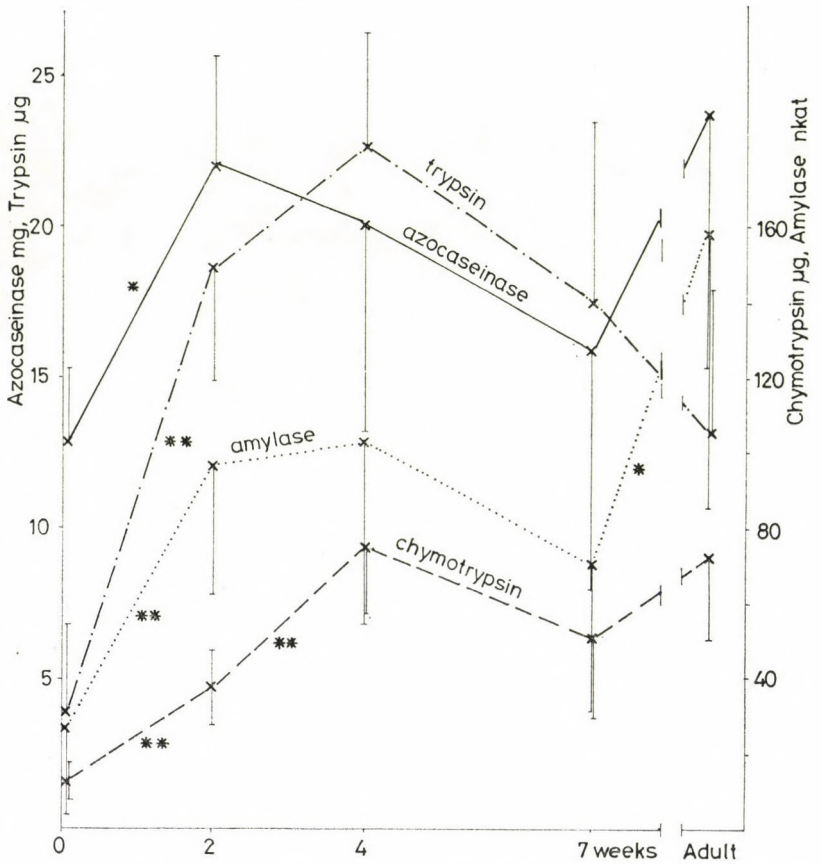

Fig. 4. Activity of azocaseinase, trypsin, chymotrypsin and amylase in the pancreas (related to mg protein ± standard deviation). Significant differences: ∗: $P < 0.05$; ∗∗: $P < 0.01$

1976) and the protease inhibitor factor of the meconium (Baintner, 1980). The latter may be secreted by the large intestinal mucosa. Ewe's colostrum has much less trypsin inhibitor than sow's colostrum and trypsin gets dominance over the inhibitor in the alimentary tract soon after birth, usually still before 12 hours of age (Baintner, 1976). In the present experiment, therefore, we could not detect free trypsin inhibitor in the small intestine, although trypsin-bound inhibitor could remain undetected.

The average *live mass* showed a slow but steady increase (Fig. 2). With advancing developmental state the natural feed of the animals changed and this directly affected the rumen content. The rumen contained only colostrum in the newborn animals, and progressively less milk and more roughage in the two-, four- and seven-week-old ones. In the latter, only roughage could be found in the rumen content, although the lambs still suckled.

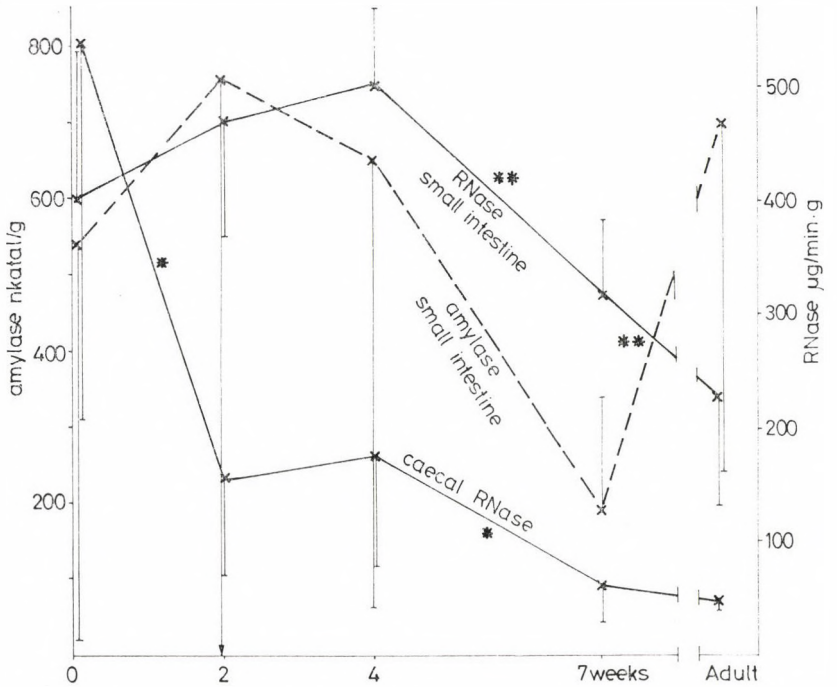

Fig. 5. RNase and amylase activities in the intestinal contents ± standard deviation. Significant differences: * $P < 0.05$; ** : $P < 0.01$

High-quality alfalfa hay was fed to lactating ewes and growing lambs and medium-quality hay to the non-producing adult animals. This difference might be reflected in higher RNase and azocaseinase activities and lower pH in the rumen content of 7-week-old lambs than in the adult animals (Fig. 1), indicating higher microbial concentration and faster fermentation in the young.

Low but definite *RNase* activity was already present in the *pancreas* of the newborn lambs (Fig. 3). Ovine values surpassed those of the pig (Baintner and Farkas, 1989) at every age.

Similar S-shape time-courses were noted in both species: a marked increase in the early postnatal period, a stagnation and finally another elevation before reaching adult values.

According to data published by Barnard (1969), ovine adult pancreatic RNase activity is 13.5-fold higher than that of the pig. By comparing values from the present work to those from an earlier study performed on pigs (Baintner and Farkas, 1989), a 26-fold interspecies difference was found between the adults, while only a 4-fold difference was demonstrable between the newborn animals. So the time-course of ovine pancreatic RNase shows a much steeper elevation than that of the pig.

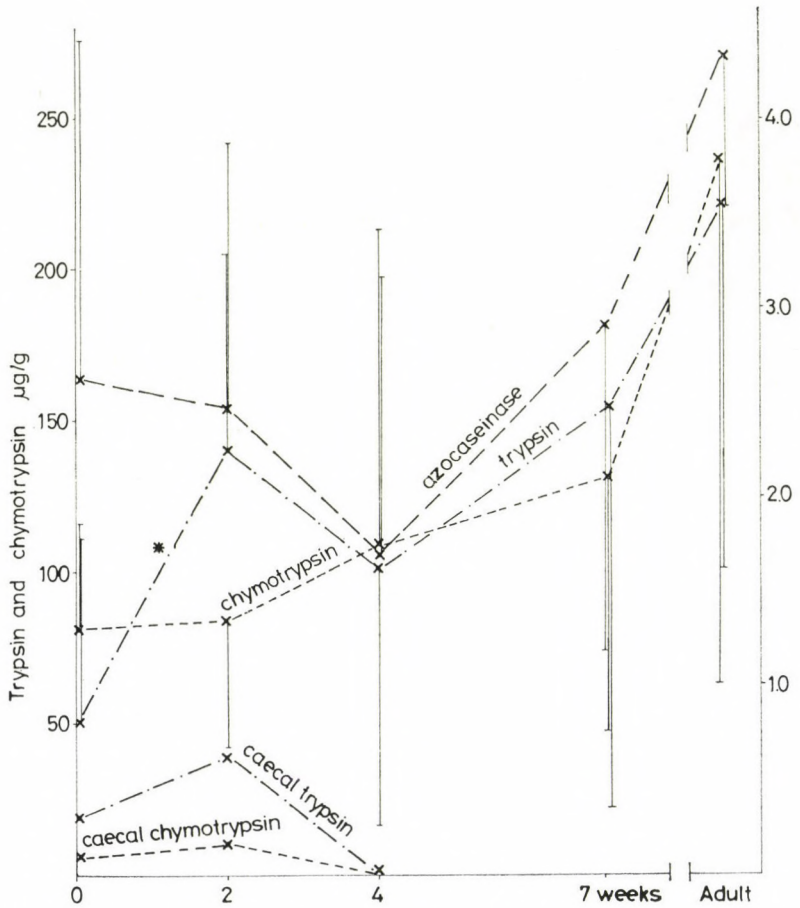

Fig. 6. Trypsin, chymotrypsin and azocaseinase activities in the small intestinal and caecal contents ± standard deviation (*: $P < 0.05$)

Development of pancreatic RNase had a similar *time-course* to pancreatic amylase and chymotrypsin. Pancreatic trypsin and azocaseinase activities also showed some similarity. All these activities increased significantly during the first two weeks of life and showed an unexplained, non-significant decrease at 7 weeks. The time-course of pancreatic lipase, intestinal enzymes and other parameters measured in the gastrointestinal tract clearly differed from that of pancreatic RNase.

Pancreatic and small intestinal RNase activities showed different time--courses; the latter had a maximum at 4 weeks, which corresponded to a minimum of small intestinal proteolytic activity (Fig. 6). We conclude, therefore, that decrease of small intestinal RNase activity after 4 weeks is the result of progressive improvement of protein digestion in older lambs and in the adult.

A similar time-course of small intestinal RNase activity was found in the pig (Baintner and Farkas, 1989), but with a maximum at 1 week.

Small intestinal and especially *caecal RNase* (Fig. 5) had high activity in the newborn. We cannot assess the contribution of bacterial RNase in the small intestine. *Rumen RNase* (Fig. 1) activity might reflect microbial growth and fermentation rate with negligible contribution by plant RNases. Improvement of rumen fermentation decreases nutrient concentration and bacterial growth in the caecum, which explains decreasing tendencies of caecal RNase with advancing age (Fig. 5).

In contrast to pancreatic and intestinal proteases of the pig (Baintner and Farkas, 1989), ovine small intestinal *trypsin: chymotrypsin ratio* did not show a tendency to increase during development. In the pancreas the ratio was inconsistent.

In agreement with our findings, Huber et al. (1961) found significant elevation of pancreatic proteolytic and amylase activities in calves in the first week of life. However, they found the same elevation for lipase, too, while we did not. Time-courses found by Walker (1959*b*) for ovine pancreatic and small intestinal proteolytic activities do not agree with our data.

Serum RNase is not included in the present paper, because turbidity that developed in the samples during incubation, resulted in erroneously high RNase values. Abomasal acidic proteolytic activity was also omitted due to a technical error.

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DETERMINATION OF GLYCOHAEMOGLOBIN LEVELS IN KETONURIC AND NON-KETONURIC COWS

(SHORT COMMUNICATION)

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(Received October 2, 1988)

Glycohaemoglobin (GHb) was estimated in haemolysate from dairy cows by colorimetric (thiobarbituric acid reaction) and ion-exchange (batch chromatographic) methods.

The percentage of the non-enzymatic glycosylation of red blood cell haemoglobin depends on the blood sugar level. In consequence of the low blood sugar level of bovines, bovine GHb exhibits a lower value than that of the other mammals. GHb level was 2.18% ($\pm$0.73) and 2.39% ($\pm$1.03) in ketonuric and non-ketonuric cows, respectively.

The authors suggest that the lower GHb level of cows with subclinical ketosis (ketonuria) is an indicator of the animals' energy-deficient status in the previous weeks.

Keywords: Batch chromatography, bovine glycohaemoglobin, ketonuria, thiobarbituric acid reaction.

According to its elution behaviour on a cation exchange resin, adult human haemoglobin (HbA) can be divided into subfractions. A few minor fast haemoglobin fractions ($HbA_{1a,\ 1b,\ 1c,}$) appear before the main component (HbA_0). These findings were first reported in the late 'fifties by Allen et al. (1958). According to results obtained later, an electrophoretically less positive haemoglobin fraction can be detected in elevated quantity in diabetic patients (Rahbar et al., 1969). Of the chromatographically fast fractions, HbA_{1c} was identical with this diabetic fraction. The chemical nature of this haemoglobin component was elucidated by structural analysis. It is a glycoprotein glycohaemoglobin (GHb) containing a glucose molecule on the N-terminal of the beta-globin chain (Bunn et al., 1975). Bunn et al. (1976) found that the glycosylation of haemoglobin takes place by non-enzymatic processes in the mature red blood cells. The percentage of this post-translational glycosylation depends on the blood sugar level (Koenig and Cerami, 1975).

As the life span of GHb is equal with that of the red blood cells, its determination is a powerful tool for retrospective evaluation of the blood sugar status. For this reason, GHb measurement is in the focus of interest of human diabetologists. This explains why data of the literature pertain mostly to humans. Only few reports are available on non-human species. These investi-

gations have been conducted on laboratory (diabetes-model) animals (Enoki et al., 1986; Higgins et al., 1982; Koenig and Cerami, 1975; Vialettes et al., 1982; Yue et al., 1982).

In our opinion determination of the GHb level can be a valuable aid in evaluating the chronic energy deficiency (critically low blood sugar level) of cows.

The studies were performed on dairy (Holstein-Friesian and Hungarian Fleckvieh × Holstein-Friesian F_1) cows of different large farms. Venous blood samples were taken from cows into heparinized tubes. The urine of the cows was tested with sodium nitroprusside ($Na_2Fe(CN)_5NO$) for ketone bodies. The centrifuged packed cells were washed with saline three times, and subsequently they were haemolysed with a detergent solution (0.3 mM sodium dodecyl sulfate). GHb determinations were made from the haemolysates either by the modified thiobarbituric acid (TBA) method with test kit (Reanal, Budapest, Hungary) or by cation exchange batch chromatography on CM--Sephadex A-50 (Pharmacia, Uppsala, Sweden) (Bárdos and Oppel, 1987).

The results obtained from the bovine GHb measurements are summarized in Table I. The level of bovine GHb is near to 2.5% of the total haemoglobin. This is half of the human value (4.5–6.5%; Miedema and Casparie, 1984). The life span of red blood cells of humans is around 120 days (Wintrobe, 1953), and that of bovine RBCs is 135–162 days (Kaneko and Cornelius, 1971). The glucose exposure period of the red blood cell haemoglobin in these ruminants is similar to that in humans. For this reason, the lower bovine GHb concentration can be explained by their lower blood sugar level.

Table I

Glycohaemoglobin content of red blood cell haemoglobin in ketonuric and non-ketonuric cows[1]

Sodium nitroprusside test of urine	Cows	
	positive	negative
No. of cows	36	125
GHb, $\%\bar{x}$	2.18	2.39
$\pm$SD	0.73	1.03

[1] Values obtained by TBA and batch chromatographic methods, respectively

In cattle, the physiological blood sugar level is one-third (1.6–3.3 mmol/l) of that measured in other mammals (Richter and Werner, 1963; Bergman, 1977) This is a phylogenic property originating from carbohydrate fermentation in their rumen. For this reason, when the gluconeogenetic pathways producing blood sugar are hindered, the quantity of ketone bodies will increase easily. This results in a keto-acidotic status, the so-called ketosis (Bergman, 1977). Ketotic animals lose their appetite and their milk production

decreases. Ketonuria is an indicator of intensive ketone body production. This condition occurs if the easily oxidizable blood sugar is not sufficient for energy production required for the metabolism. Our results show that ketonuric cows have a lower GHb level than non-ketonuric ones. In spite of the lack of previous blood sugar measurement, this lower GHb level indicates that the blood sugar level of these cows was low in the last two months.

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VARIABILITY OF TOOTH ERUPTION IN CATTLE

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Absolute and relative ages of incisor and canine eruption were recorded in 152 Holstein-Friesian and 154 Limousine × Hungarian Fleckvieh cows kept under commercial circumstances. While significant difference was found only between the emergence of the first incisor relative to the canine, a broader comparison of several breeds as published by a variety of authors put the findings within the right context. Results of a discriminant analysis suggested that the absolute ages of tooth eruption are heavily influenced by the environment. Typological inferences may be made studying tooth eruption ages relative to each other. In archaeozoology, aging based on modern toothwear analogies may be biased by these two inseparable effects.

Keywords: Cattle aging, tooth eruption, maturation types, discriminant analysis

Observation of tooth eruption and toothwear in various domestic animals has been of interest since classical times. Commercial cattle breeding and beef industry, however, developed more business-oriented systems for evaluating optimal stages for manipulating the lives of animals from weaning until slaughter. Both traditional aging based on dental development and modern rearing technologies using weight data focus on biological age.

The comparison of the two methods of aging using absolute age as a common denominator is not totally effective when only tooth eruption data are available because permanent teeth usually appear in the oral cavity when the first, most dynamic increase of liveweight has already ended. Thus, overall quantitative growth data are not easily convertable into tooth eruption patterns.

The aim of this paper is to isolate characteristics of growth intensity in two breeds of different purpose on the basis of both methodologies.

Although this article is part of a wider ontogenetic study concerning cattle, results discussed here may be of particular interest in archaeozoology, where quite often the only evidence available for reconstructing kill-off patterns is the analysis of toothwear in the faunal material. This work, however, is very much dependent on modern analogies as is shown by tendencies of the most progressive research in this topic (e.g. Payne, 1973).

Toothwear itself is a result of a very complex process involving both environmental and genetic factors. Tooth eruption is of basic importance as it

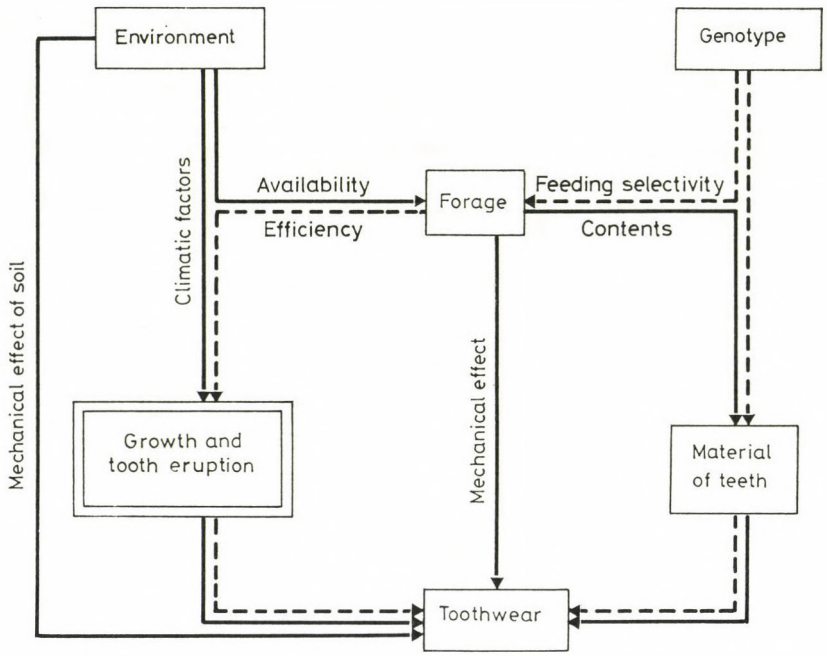

Fig. 1. The effects of genotype (dashed lines) and environment (continuous lines) on tooth eruption and toothwear respectively

represents the *beginning* of toothwear and may interact in many ways influencing even relative aging carried out by toothwear analysis. These inter-connections are shown in Fig. 1. This is the point at which the conclusions of this paper may contribute to the general knowledge of aging based on data derived from the condition of the dental system.

Hypothesis and test implications

The data of this study were used to test within species variability of tooth eruption. Aside from the two breeds compared here, the results were also analyzed against a background of a large series of data from the literature to test the following problems:

1. How general growth and development characteristics are reflected in the age of eruption for the teeth studied (tested by the consideration of liveweight growth data and inter-breed comparison using univariate statistics).

2. Are there statistically separable maturity groups to be distinguished (including the two breeds under study here) which correspond to the practical

classification of Habermehl (1961), tested by a stepwise discriminant analysis (Jennrich and Sampson, 1977).

The test implication for the applied calculations was a $p \leq 0.05$ level of significance for differences between mean values.

Materials and methods

This study is based on the tooth eruption patterns of 152 cows studied at the Experimental Farm of the Hungarian Academy of Sciences, Martonvásár, and 154 cows from the "Táncsics" Agricultural Co-operative, Mezőhék. The first group consisted of Holstein-Friesian dairy cattle, while the second included individuals from the first generation of the beef-oriented crossing of dual-purpose Hungarian Fleckvieh and Limousine cattle. As far as this sample was concerned, only female cattle were used. Unfortunately, the heterogeneity of data derived from the literature also did not provide a basis for testing sexual dimorphism (Habermehl, 1961; Brown et al., 1960; Schmid, 1972; Horn, 1976) potentially manifested in tooth eruption. In this respect one has to rely on the conclusion of Brown et al. (1960) who found no consistent differences in the chronology of tooth development between cows and bulls using a sample of 869 animals.

"Anterior teeth" (Graham and Price, 1982) of cattle provided the main focus of this analysis.

The dental formula for permanent teeth in cattle is as follows:

$$\frac{0033}{3133}$$

The six incisors of cattle appear only in the mandible and form a continuous row with the canine which is often considered the "fourth" incisor. This tooth in cattle, however, is a true canine which since the Oligocene has changed its form and became aligned beside the third incisor (Loomis, 1925).

In the two populations, eruption of the three left incisors and canine were recorded during overall measurement work. Although the time of eruption may be different for the two sides, developmental processes occur almost simultaneously for each of a pair of incisors so that they may be treated as identical ones (Brown et al., 1960; Murphy, 1981).

The time of eruption for premolars and molars into the oral cavity is also of interest, but the accurate *in vivo* study of these teeth proved difficult i.e. seemed to be a great source of bias. This problem therefore is beyond the focus of this paper.

In addition to the ages when tooth eruption was first observed (recorded in days and months respectively), the occurrence of each incisor relative to

the time of canine eruption was calculated. This resulted in percentages showing the tooth's position within the time interval between birth and the end of teething in the oral part of the mandible.

The use of such ratio values has been criticized for potential distortions due to differences between the coefficients of variation calculated for the numerator and denominator (Atchley et al., 1976). In fact, such differences may be observed in Table I: standard deviations relative to mean values increase consistently with age as is shown by the coefficients of variation expressed in percentages. Since, however, correlations most sensitive to such bias were not studied in this paper, the informative value of such composite descriptors seemed to dominate over their disadvantages.

Results

Table I shows the comparison between tooth eruption patterns of the two groups of cattle under discussion. As shown by the results, no significant differences occurred between the two breeds in terms of the absolute ages of tooth eruption. The probability of coincidental differences in the case of these variables is slightly beyond the limit set in the test implications. The lack of significant differences, however, perfectly corresponds to observations by Tulloh (1962), who recorded the time of eruption for the first pair of permanent

Table I

Incisor and canine eruption in two breeds from Hungary

	Absolute age (days)				Relative age (%)		
	Incisor 1	Incisor 2	Incisor 3	Canine	Incisor 1	Incisor 2	Incisor 3
					Canine		
Holstein-Friesian							
n	48	25	43	36	35	25	35
$\bar{x}$ (day)	674	982	1174	1336	50.4	73.5	87.9
sd (day)	15.2	23.4	151.2	212.3	2.1	2.3	8.4
cv (%)	2.2	2.4	12.9	15.9	4.1	3.1	9.6
Limousine × Hungarian Fleckvieh							
n	36	41	39	38	36	38	38
$\bar{x}$ (day)	686	970	1104	1223	56.1	79.3	90.3
sd (day)	11.1	24.1	142.0	207.1	1.9	2.4	8.4
cv (%)	1.6	2.5	12.9	16.9	3.5	3.1	9.4
df	82	64	80	71	69	61	71
t-test, P	—	—	—	—	0.05	—	—

incisors in 36 beef steers (Shorthorn, Hereford and Aberdeen Angus respectively). The eruption of the first incisor in the oral cavity occurs slightly earlier in Holstein-Friesians, although this breed shows greater standard deviations than the other and the beef breeds studied by Tulloh (1962). The eruption of the second incisor follows practically simultaneously in the two groups, while during the rest of ontogeny the third incisor and canine occur somewhat earlier in Hungarian Fleckvieh and Limousine crosses. This latter phenomenon may be due to the fact that beef cattle are selected for especially intensive development and may also be influenced by possible heterosis in the F_1 generation under study here.

The inclusion of percentual tooth eruption data provided more marked differences. The first and second incisors erupt later in relative terms in the beef crosses. The basic pattern, however, is that of convergence between the two breeds with the advancement of ontogeny.

While ratio values are usually perceived as being less influenced by environmental effects, marked differences between the early rearing of these dairy and beef cattle under the circumstances of modern, specialized farming must be pointed out here. It is possible that ranged beef heifers catch up later with Holstein calves brought up in a more controlled environment. Consequently, while differences in tooth eruption fit the general notion that beef calves selected for a comparably smaller birth weight grow more dynamically, the phenomenon observed in the relative timing of incisor eruption may, to some extent, exemplify compensational growth, evening out environmental

Table II

Changes in the liveweight of heifers

Age (day)	Growth stages			
	1 0–120	2 121–240	3 241–380	4 381 or more
Holstein-Friesian				
n	22	16	17	27
$\bar{x}$ (kg)	56.0	160.0	298.4	409.9
sd (kg)	40.9	24.3	74.1	70.6
cv (%)	73.0	15.2	24.8	17.2
Limousine × Hungarian Fleckvieh				
n	37	19	17	21
$\bar{x}$ (kg)	56.7	181.6	291.3	452.6
sd (kg)	30.7	29.3	36.6	96.0
cv (%)	54.1	16.2	12.6	21.2
df	57	33	32	46

differences throughout early ontogeny (Winchester et al., 1957). This phenomenon, however, could only be exactly observed in a planned, large-scale experiment, rather than using a random sample of animals.

In Table II one may observe similar tendencies in the early postnatal ontogeny of female calves and young heifers. Although due to the small number of cases the majority of the differences are not significant, it is worthy of note that after a similar beginning, Holstein-Friesian calves show a less dynamic but steady growth in the four developmental stages separated on the basis of growth curves analysed during previous research (Gere and Bartosiewicz, 1979). These groups differ from those in Table I due to overlaps in tooth eruption. Standard deviations in Table II are relatively large because of the long time intervals represented by the age groups. In spite of this, a somewhat smaller variability of liveweight is observable in the case of the beef type Hungarian Fleckvieh × Limousine cross calves. This tendency is, however, not as unambiguous as in the case of the coefficients of variation of incisor (and canine) eruption.

Finally the results concerning the chronology of tooth eruption are studied in a wider, intraspecific context. Although data obtained from the literature were not always published with statistical details, it seemed reasonable to outline a framework for the above presented data using at least mean values. In this way a rich list of breeds (or in some cases forms of breeds) could

Table III

Tooth eruption data for early maturing cattle (in months)

I_1	I_2	I_3	C	Breed	Source
14.0	17.0	22.0	32.0	"early maturing"	Schumer in Habermehl, 1961
17.0	22.0	32.0	36.0	"early maturing"	Zietschmann et al., 1943
17.0	22.0	32.0	36.0	"early maturing"	Ellenberger and Baum, 1908
18.0	23.0	31.5	40.0	Red Pied Lowland	Schwenken, 1955
18.0	27.0	36.0	45.0	Black Pied Lowland	Habermehl, 1961
18.0	30.0	33.0	42.0	general	Kroom in Habermehl, 1961
18.0	30.0	42.0	54.0	general	Schönberg, 1928
19.5	21.5	31.0	40.0	general	Silver, 1963
19.5	28.5	34.5	43.5	Yellow Frankish	Schaper, 1927
20.0	26.0	33.0	42.0	Simmenthal	Habermehl, 1961
20.0	26.0	36.0	46.0	general	Schmid, 1972
20.0	28.0	37.0	44.0	Black Pied Lowland (intensive)	Manns, 1924
21.0	27.0	36.0	42.0	Black Pied Lowland	Schlüter, 1923
21.0	27.0	36.0	45.0	"medium maturing"	Zietschmann et al., 1943
21.0	27.0	36.0	45.0	"medium maturing"	Ellenberger and Baum, 1908

be compiled, offering an opportunity for a more global evaluation of the previously studied two groups. The majority of data is formed by largely traditional breeds of local importance from the territory of Germany and Poland recorded during the heyday of such odontological studies between the two world wars and summarized by Habermehl (1961). In addition to these and data of Table I, some modern breeds were also included (Tables III, IV and V).

In arranging these data, Habermehl's classification model was applied with some simplification: the data were distinguished as coming from early

Table IV

Tooth eruption data for medium maturing cattle (in months)

I_1	I_2	I_3	C	Breed	Source
21.0	29.0	37.0	46.0	Allgau Brown	Gattinger, 1927
21.0	32.0	36.0	48.0	general	Horn, 1976
21.5	27.5	33.5	38.5	Shorthorn	Kohnke, 1923
21.5	30.2	35.7	43.5	Holstein Friesian	Brown et al., 1960
22.0	28.0	36.0	45.0	Pinzgau ("late")	Gattinger, 1927
22.0	30.0	36.0	45.0	commercial cross	Miller in Silver, 1963
22.0	30.0	39.0	48.0	Vogelsberger	Gattinger, 1927
22.0	30.0	39.0	48.0	Allgau brown	Gattinger, 1927
22.0	31.0	40.0	48.0	Brown cattle	Habermehl, 1961
22.1	32.2	38.5	43.8	Holstein-Friesian	Bartosiewicz
22.6	27.5	34.5	42.7	general	Andrews, 1974
22.5	30.0	39.0	45.0	Maas-Rhein-Yssel Red Pied Lowland	Plank, 1922
22.5	31.8	36.2	40.1	Limousine and Hungarian Fleckvieh cross	Bartosiewicz
23.0	28.0	36.0	43.0	Black Pied Lowland	Heldt, 1922
23.0	29.0	38.0	45.0	Hinterwalder	Habermehl, 1961
23.0	31.0	39.0	48.0	Simmenthal	Gattinger, 1927
23.0	33.0	42.0	57.0	ranch cattle	Miller in Silver 1963
23.1	31.0	34.8	41.0	Hereford	Brown et al. 1960
23.5	29.0	35.5	44.5	Angler ("medium")	Kohnke, 1923
23.6	30.0	35.0	42.0	Guernsey	Brown et al., 1960
24.0	28.5	36.5	49.0	Schleswig Holstein Red Pied Lowland	Habermehl, 1961
24.0	30.0	36.0	41.0	Aberdeen Angus	Brown et al., 1960
24.0	30.0	38.0	45.0	Harz cattle	Schaper, 1927
24.0	30.0	38.0	48.0	Pinzgau	Winkelmann, 1948
24.0	30.0	42.0	48.0	Fleckvieh ("medium")	Habermehl, 1961
24.0	32.0	40.0	49.6	mean of five breeds	Graham and Price, 1982
24.0	33.0	39.0	50.0	Brown Swiss	Glauss, 1932
24.0	33.5	40.5	50.5	Black Pied Lowland	Manns, 1924
24.0	36.0	48.0	55.0	Middle German	Habermehl, 1961
25.0	31.0	40.0	47.0	Frankish	Schaper, 1927

maturing, medium and late maturing groups based on the averages of the maximum and minimum values recorded for each group. In order to test some possible deviations from Habermehl's classification and locate the two breeds discussed in this study a stepwise discriminant analysis was performed.

Table V

Tooth eruption data for late maturing cattle (in months)

I_1	I_2	I_3	C	Breed	Source
25.0	32.0	40.0	52.0	"late maturing"	Zietschmann et al., 1943
25.0	32.0	40.0	52.0	"late maturing"	Ellenberger and Baum, 1908
25.0	32.0	41.0	51.0	Harz cattle (extensive)	Schaper, 1927
25.0	33.0	40.0	42.0	"late maturing"	Schummer in Habermehl, 1961
25.5	33.0	42.0	49.0	Rhine cattle ("medium")	Habermehl, 1961
26.0	32.0	40.0	46.0	Weser marsh cattle	Franksen, 1941
26.0	32.0	42.0	48.0	Black Pied Lowland	Habermehl, 1961
28.0	35.5	43.0	51.5	Black Pied East Friesian	Vogelsang, 1948
29.5	35.0	46.5	57.0	Black Pied Lowland (extensive)	Manns, 1924
31.2	44.0	59.6	65.9	Hooghly zebu	Obend'hal, 1981
50.3	66.7	75.2	88.0	Purulia zebu	Obend'hal, 1981

Table VI

Incisor and canine eruption in three maturation groups of cattle (Habermehl's criteria)

	Absolute age (months)				Relative age (%)		
	Incisor 1	Incisor 2	Incisor 3	Canine	Incisor 1	Incisor 2	Incisor 3
					Canine		
Early maturing n = 15							
$\bar{x}$	18.6	25.6	34.4	42.9	44.1	60.1	80.2
sd	1.9	3.7	4.6	5.8	4.9	4.8	4.9
cv (%)	9.9	14.3	13.5	13.5	11.0	7.9	6.1
Medium maturing n = 30							
$\bar{x}$	22.8	30.5	37.8	46.1	49.9	66.4	82.3
sd	1.0	1.9	3.0	4.2	4.5	4.9	4.3
cv (%)	4.5	6.5	7.9	9.1	9.1	7.4	5.2
Late maturing n = 11							
$\bar{x}$	28.8	37.0	46.3	54.8	52.6	67.4	84.6
sd	7.4	10.5	11.1	12.6	4.1	5.8	5.6
cv (%)	25.8	28.5	24.0	23.0	7.9	8.6	6.6

Table VI summarizes the mean values, standard deviations, and coefficients of variation for the three groups (Holstein-Friesian cows and Limousine crosses both occur in the "medium" group). On the basis of within sample variations only modern Holstein-Friesians and some forms of brown highland cattle were re-grouped as medium forms (clustered with Highland Fleckvieh, German Red and Angler cattle for example) as opposed to breeds considered early maturing by Habermehl (Red and White Lowland cattle, Shorthorn, Simmental, etc.). It is quite possible, however, that the inclusion of modern breeds kept under more intensive circumstances shifted the lower limit of the medium group toward earlier maturation.

The process of distinguishing between the three maturation groups under study began with the recognition of which tooth has the greatest discriminating power (Table VII). In order to select such variables F tests were calculated and the teeth showing the highest F value were identified as incisor 1 (both in terms of absolute age and relative to canine eruption) and the age at which canine occurred. At the same time, a U statistic was calculated to test the equality of group means.

The F value indicates the importance of the relationship between the first incisor's and the canine's time of eruption in distinguishing between cattle forms on the basis of incisor and canine development respectively.

Final conclusions were based on the classification functions by which the division obtained in this study corresponds to the maturation groups defined by the criteria of Habermehl (1961) in 85.7% of the cases (Tables VIII

Table VII

The selection of discriminating variables

Step	Variable used	F value to enter	U statistic	df	
1	incisor 1	26.856	0.492	2	52
2	incisor 1/canine	4.422	0.419	4	102
3	canine	11.624	0.286	6	100

Table VIII

Classification functions

Coefficients for	Groups by maturation		
	Early	Medium	Late
Incisor 1	−149.8	−153.7	−154.6
Canine	82.8	85.1	85.9
Incisor 1/Canine	83.9	86.4	87.3
Constant	−2225.9	−2364.2	−2420.6

Table IX

Jackknifed classification matrix

Number of individual breeds	Groups obtained			Criteria by Habermehl 1961	Percent correct
	Early	Medium	Late		
Early maturing	14	1	0	15	93.3
Medium maturing	2	27	1	30	90.0
Late maturing	0	4	7	11	63.6
Total	16	32	8	56	86.7

and IX). This slight discrepancy is in part due to the fact that relative tooth eruption data were also taken into consideration.

The calculations were carried out using a so-called "jacknifed classification" in which D^2 values were computed for the distance from each case to the groups formed by the remaining individual breeds.

In terms of the distinction based on the three variables of most discriminating value, the Black Pied Lowland cattle recorded by Schlüter (1923) was re-classified as medium maturing. On the other hand, two members of the medium maturing group, Allgau Brown by Gattinger (1927) and the general norm published by Horn (1976) more recently, would be considered early maturing in Habermehl's 1961 classification which, in part, is based on traditional, if not primitive breeds. Within the medium maturing group only Aberdeen Angus cattle, as described by Brown et al. (1960), would fall into the late maturing group on the basis of its tooth eruption schedule characterized by a short interval between the occurrence of the first incisor and the canine teeth. In the least clearly defined group of late maturing cattle, the early eruption of the first incisor relative to the canine tooth places the Harz (Schaper, 1929) and Rhine (Habermehl, 1961) breeds among the medium maturing cattle together with the general norms for late dental maturation suggested by the early work of Ellenberger and Baum (1908) as well as Zietschmann et al. (1943). Correspondingly, the percentage of correct classifications is lowest in this third group.

Conclusions

General remarks

Holstein-Friesians and the Limousine breed crossed with Hungarian Fleckvieh analysed in the first part of this study are firmly positioned within the group of medium maturing cattle. While these two modern breeds display no dramatic differences in tooth eruption under the circumstances character-

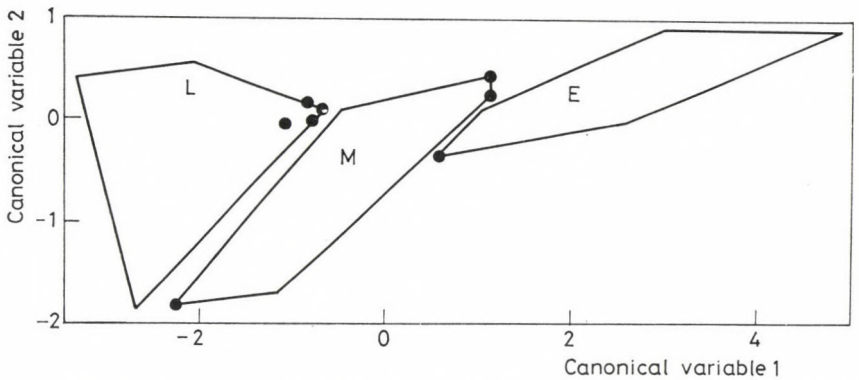

Fig. 2. The outline of early (E), medium (M) and late (L) maturing groups according to Habermehl's classification (1961). The letters stand for group means, data points symbolize misclassified cases

istic of commercial rearing, they deserve attention as part of a broadly based intraspecific comparison.

Results of the discriminant analysis are summarized in Fig. 2 which is based on the scattergram of two canonical variables expressing the relationships between tooth eruption data and the codes labelling the early, medium and late maturation groups into which the sample of cattle breeds was sub-divided. It is noteworthy that minor deviations from Habermehl's classification based on absolute tooth eruption chronologies alone, occur along the X axis (canonical variable 1) in the form of overlaps at the extreme ends of the medium maturing group. These "incorrect" classifications may be related to the inclusion of more modern cattle breeds in addition to a number of breeds recorded in Central Europe during the 1920's. In Fig. 3, data points representing three different

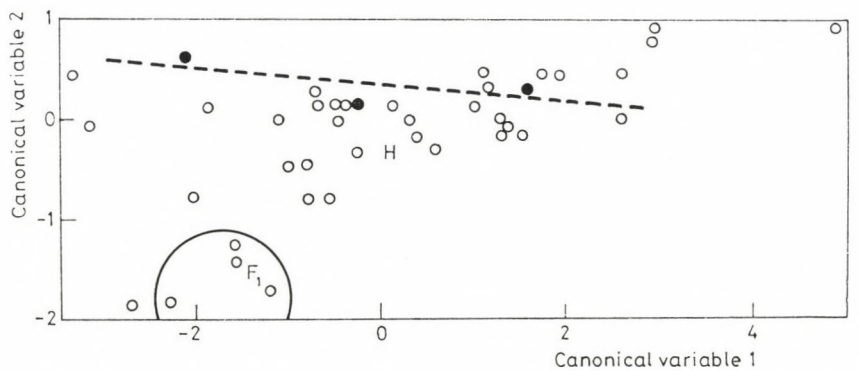

Fig. 3. The position of Holstein-Friesians (H) and crosses between Limousine and Hungarian Fleckvieh (F_1) within the configuration of data points. Full circles and dashed line show the effect of decreasing levels of nutrition on Black Pied Lowland cattle (right to left; Manns, 1924). Dynamically growing (beef) breeds are encircled

planes of nutrition in Black Pied Lowland cattle in the Mazur region (Manns, 1924) each fall into one of the three maturation groups respectively, thus forming a clear trend that runs parallel with X axis (first canonical variable). Consequently, distribution of the first canonical variable seems to be strongly influenced by the modes of keeping and environment in general.

Specialized types

The eruption of the first incisor relative to the canine, however, added a new dimension to the data set, represented by the second canonical variable. While the range along the Y axis associated with this trait is smaller and thus more difficult to analyse, the extremes deserve particular attention. The highest values of Y are related to early recordings of dental maturation (Ellenberger and Baum, 1908), data on probably more primitive breeds such as the previously mentioned Black Pied Lowland cattle between the two world wars (e.g. Manns, 1924), and the slowly maturing zebu (Obend'hal, 1981). Extreme negative values, on the other hand, occur around the F_1 generation beef crosses studied from Hungary (Limousine and Hungarian Fleckvieh) and include Hereford (Brown et al., 1960), Shorthorn (Kohnke, 1923) as well as early maturing Guernsey cattle (Brown et al., 1960). The case of the "misclassified" Aberdeen Angus breed (Brown et al., 1960) further supports the observation that distribution along the Y axis shows more genetically determined early maturation than its manifestation related to environmental circumstances (Bartosiewicz et al., 1987). The distribution of data points in the plane defined by these two canonical variables, however, expresses the massive effect of environment on absolute age when teeth erupt.

The requirements of modern animal husbandry cause a decrease in the variability of specialized populations. This is a conscious effort in the selection on the basis of production characteristics, but also results in the evolution of qualities which are not directly effected by the breeding work (Lauvergne, 1975). The head and dental structure, on the other hand, are considered to change slowly relative to other traits directly exposed to conscious selection. Thus, tooth eruption schedules of the small group of dynamically growing (mostly beef) cattle support the point raised by Graham and Price (1982) that dental age can be objective and observable in the live animal, yielding a more precise estimate of chronological age and has the potential to replace physiological age estimation for beef carcasses. Brookes and Hodges (1979) claim to have observed a significant breed effect in the eruption of the first incisor (Hereford 36 days later than dairy Shorthorn), although the level of nutrition was found to have at least as great effect as breed. The same difference between these two breeds was reported by Tulloh (1962), while Hereford was not different from Aberdeen Angus in that study.

In less specialized forms and under less intensively industrialized circumstances a greater heterogeneity may be expected such as was seen with zebu ranged by traditional methods in two ecological regions of India (Obend'hal, 1981).

Archaeozoological aspects

Conclusions for applied archaeozoological research should be based on the realization of tooth eruption's importance in shaping the final appearance of toothwear. This latter greatly depends on the duration of use to which the tooth was exposed (Murphy, 1969). With the inclusion of a number of cattle breeds, a great variability of tooth eruption patterns was observed which indirectly influence final toothwear, usually considered to be indicative of absolute age.

Although in this paper dentition chronologies of cattle of different purposes were found to be different, and such information should be of great importance for archaeological interpretation (Payne, 1973), these characteristics did not reflect directly the growth tendencies of liveweight. Knowledge of this latter, however, is of basic importance for relative aging. The direct correlation between dentition and body weight was observed only when the presence, i.e. eruption, of certain teeth as a quasi continuous quantitative character (Fábián, 1965) was studied relative to changes in body weight. Thus, the lack or presence of lower third molars has been correlated with body weight in mice (Grüneberg, 1951). Such extreme changes, however, have not been studied intensively in cattle under experimental circumstances.

Even the random data set compiled for this paper raises an important point. In light of the influence of evolutionary and regional effects on tooth eruption (and other factors affecting toothwater subsequently), dental aging based on modern analogies may be misleading in archaeozoology. After the fifth year of age, dental ages become unreliable in red deer stags (Bán and Szidnai, 1987), which is a genetically more homogeneous species than any of the domestic Artiodactyls. Lowe (1967) used molar wear stages for red deer as well as found them useful "to some effect" for aging these game animals. These examples illustrate how essential cautiousness is when dental ages from different periods and archaeological sites are compared.

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STUDY OF THE TOXIN-PRODUCING ABILITY OF *PASTEURELLA MULTOCIDA* IN MICE

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Cell-free sonicated extracts and broth cultures of *Pasteurella multocida* strains of pig origin were examined for their lienotoxicity in mice. *P. multocida* strains represented capsular types A and D with or without dermonecrotoxic (DNT) activity in the guinea pig skin test.

Mouse lienotoxicity test was suitable for determining the toxigenicity of *P. multocida* strains only when bacterium-free extracts were tested. In that case both toxigenic type A and D strains were lethal to intravenously inoculated mice and caused a remarkable reduction in spleen mass when sublethal doses were used. The extracts of atoxic strains were not lethal and induced splenic hyperplasia.

By testing viable cells no correlation was demonstrable between toxin production and virulence of *P. multocida* to mice.

In one experiment the concentrated sterile culture fluids of a toxigenic type D *P. multocida* and a toxigenic *B. bronchiseptica* strain were compared. The former caused deaths and splenic atrophy among mice, while the latter was nontoxic and induced slight hyperplasia of the spleen. This fact indicates that *P. multocida* secretes its toxin into the culture fluid.

Keywords: *Pasteurella multocida*, toxin, lienotoxicity, mouse

From data of the literature it is obvious that toxigenic *Pasteurella multocida* strains play an important role in the aetiology of atrophic rhinitis (AR) of swine (Pedersen and Barfod, 1981; Rutter and Rojas, 1982; Chanter et al., 1986). There are different methods for determining the toxin-producing ability of *P. multocida:* the guinea pig skin test (de Jong et al., 1980), toxicity to embryonic bovine lung cells (Rutter and Luther, 1984), and lethality in intraperitoneally inoculated mice (Rutter, 1983). All these methods employ bacterium-free sonicated extracts of *P. multocida.*

Éliás et al. (1986) recommended a mouse lienotoxicity test for this purpose. They found that broth cultures of toxigenic *P. multocida* strains were lethal to mice a few hours after intravenous injection. The spleens of mice surviving up to postinoculation day 7 remained almost unaltered. On the other hand, inoculation with atoxic strains resulted in marked splenic hyperplasia.

The aim of the present study was to compare the effects of *P. multocida* broth cultures and cell-free sonicated extracts on mice following intravenous administration.

Materials and methods

Bacterial strains

Eight *P. multocida* strains and one *Bordetella bronchiseptica* strain isolated from pigs were used. *P. multocida* LFB3 (toxigenic type D) and *B. bronchiseptica* GF8 were kindly supplied by Dr. J. M. Rutter (Compton, U.K.). *P. multocida* M444 (atoxic type D), M297 (toxigenic type A) and M439 (atoxic type A) were kindly provided by Dr. P. Kielstein (Jena, GDR). *P. multocida* strains designated P71, P72, P81 and P108 were isolated from different pig herds in Hungary. Type A strains were identified by their inhibition of capsule formation when growing near a hyaluronidase-producing strain of *Staphylococcus aureus* (Carter and Rundell, 1975). Type D strains were identified by their autoagglutination in the presence of a 1 : 1000 solution of acriflavine neutral (Carter and Subronto, 1973).

Preparation of cell-free sonicated extracts

P. multocida strains were propagated on blood agar plates containing 5 per cent sheep blood and supplemented with 1% yeast extract. The plates were incubated at 37 °C for 18 h. The bacterial growths were washed twice in phosphate buffered saline (PBS) and after centrifugation the pellets were resuspended in distilled water to a dilution of one in ten (w/v). The bacterial suspensions were sonicated at 1.5 A for 2×1 min with an interval of 1 min, during cooling in ice-bath. The sonicates were then centrifuged at 12.000 x *g* for 60 min at 4 °C and the clear supernates were filtered through a 0.22 μm membrane filter (Millipore Corp., Bedford).

In one experiment *P. multocida* LFB3 and *B. bronchiseptica* GF8 were cultivated in brain heart infusion (BHI) broth (Difco, Detroit) at 37 °C for 18 h. The cultures were centrifuged and cell-free sonicated extracts were prepared from the pellets by the method described above. Moreover, the supernates of the original BHI broth cultures were also filtered through a 0.22 μm membrane filter and the filtrates were concentrated about 10-fold by dialysis against polyethylene glycol 20,000 (Fluka AG, Basel).

Guinea pig skin test

To test for dermonecrosis, previously depilated guinea pigs weighing 300 to 350 g were inoculated intradermally with 0.1 ml of one of the bacterial extracts. Dermonecrotic lesions $>$ 5 mm were recorded after 48 h.

Mouse experiments

Inbred albino mice of CFLP strain (LATI, Gödöllő) of both sexes, 16 to 18 g in body mass, were used.

In the first experiment groups of five mice were injected into the lateral tail vein, with 0.2 ml of one of serial 10-fold dilutions (10^{-1} to 10^{-4}) of 18 h *P. multocida* BHI broth cultures (Éliás et al., 1986).

In the second experiment groups of five mice were inoculated intravenously with 0.2 ml of one of serial 3-fold dilutions of *P. multocida* cell-free sonicated extracts.

In the third experiment groups of five mice were inoculated intravenously with 0.2 ml of one of serial 3-fold dilutions of *B. bronchiseptica* GF8 and *P. multocida* LFB3 extracts prepared from BHI broth culture pellets and the filtered and concentrated BHI broth culture supernates, respectively.

In all experiments deaths were recorded up to postinoculation day 7, then the survivors were killed and their spleen masses were determined. Relative spleen mass was calculated for 10 g body mass in each case.

Results

The results of capsule typing and guinea pig skin test as well as those of mouse lienotoxicity test with BHI broth cultures of *P. multocida* are shown in Table I. Intravenous inoculation of mice with serial 10-fold dilutions of broth cultures of the different *P. multocida* strains resulted in a varying rate of

Table I

Mouse lienotoxicity test with broth cultures of *P. multocida*

Strains	Capsule type	DNT	Dilution of 18-h BHI broth cultures							
			10^{-1}		10^{-2}		10^{-3}		10^{-4}	
			let	sm	let	sm	let	sm	let	sm
LFB3	D	+	5/5	—	0/5	75*	0/5	92	0/5	79
P108	D	+	5/5	—	3/5	87	0/5	83	0/5	54
P81	D	—	3/5	66	0/5	82	0/5	72	0/5	60
M444	D	—	5/5	—	0/5	104	1/5	75	0/5	64
M297	A	+	1/5	81	0/5	81	0/5	106	0/5	90
P71	A	+	5/5	—	5/5	—	0/5	63	0/5	48
M439	A	—	3/5	64	0/5	78	0/5	70	0/5	85
P72	A	—	5/5	—	5/5	—	5/5	—	3/5	95

* relative spleen mass of control mice: 55; DNT: dermonecrotoxin detected in guinea pig skin test; BHI: brain heart infusion; let: lethality (number of mice that died/number of mice in the group); sm: relative spleen mass

Table II

Mouse lienotoxicity test with cell-free sonicated extracts of *P. multocida*

Strain	Dilutions of extracts											
	UD		1 : 3		1 : 9		1 : 27		1 : 81		1 : 243	
	let	sm	let	sm	let	sm	let	sm	let	sm	let	sm
LFB3 (toxic D)+	ND	ND	5/5	—	5/5	—	5/5	—	3/5	21*	0/5	43
P108 (toxic D)	ND	ND	5/5	—	5/5	—	4/5	17	0/5	34	0/5	50
M444 (atoxic D)	0/5	101	0/5	100	0/5	70	0/5	54	ND	ND	ND	ND
P81 (atoxic D)	0/5	78	0/5	65	0/5	59	0/5	56	ND	ND	ND	ND
M297 (toxic A)	ND	ND	5/5	—	5/5	—	5/5	—	2/5	17	0/5	32
P71 (toxic A)	ND	ND	5/5	—	5/5	—	5/5	—	3/5	11	0/5	37
M439 (atoxic A)	0/5	85	0/5	67	0/5	59	0/5	57	ND	ND	ND	ND
P72 (atoxic A)	0/5	117	0/5	98	0/5	83	0/5	69	ND	ND	ND	ND

UD: undiluted; ND: not determined; let: lethality (number of mice that died/number of mice in the group); sm: relative spleen mass; *relative spleen mass of control mice: 55; +dermonecrotoxicity in guinea pig skin test and capsular type

mortality. No close correlation was demonstrable between the mortality rate and DNT production of the strains. *P. multocida* strain P72 (atoxic type A) was much more lethal than strain M297 (toxic type A). The DNT-positive strain LFB3 and the DNT-negative strain M444 were similar as regards lethality. For all strains but P71 a varying degree of increase was found in relative spleen mass, and no correlation was demonstrable between DNT-producing ability and the change in spleen mass as compared to the control.

On the other hand, among groups of mice inoculated with *P. multocida* crude extracts significant differences occurred both in lethality and in the change of spleen mass (Table II). In lower dilutions the extracts of the toxic

Table III

Mouse lienotoxicity test with the supernates and cell-free sonicated extracts of toxigenic *B. bronchiseptica* and *P. multocida* broth cultures

Dilution	*B. bronchiseptica* GF8				*P. mutlocida* LFB3			
	Extract		Supernate		Extract		Supernate	
	let	sm	let	sm	let	sm	let	sm
1 : 3	5/5	—	0/5	74	5/5	—	5/5	—
1 : 9	3/5	21	0/5	61	5/5	—	5/5	—
1 : 27	0/5	44	0/5	63	5/5	—	2/5	18
1 : 81	0/5	54	0/5	57	4/5	11	0/5	31
1 : 243	0/5	57	0/5	60	2/5	14	0/5	61

let: lethality (number of mice that died/number of mice in the group); sm: relative spleen mass

strains (both type A and type D) rapidly caused death. The spleen mass of mice surviving inoculation with higher dilutions of the extracts substantially decreased. Extracts of the non-toxigenic strains did not cause deaths but they increased the spleen mass of the inoculated mice in a dose-dependent manner.

Table III shows the results obtained by intravenous inoculation of mice with sterile concentrated supernates of BHI broth cultures of a toxigenic *B. bronchiseptica* and *P. multocida* strain and with crude toxins prepared from the sediments by sonication. The sonicated cell-free extracts of both strains exerted similar effects on mice. They were lethal in lower dilutions and caused marked atrophy of the spleen in the surviving mice. The only difference was that *P. multocida* LFB3 was more toxic than *B. bronchiseptica* GF8. On the other hand, the sterile filtrate of *B. bronchiseptica* GF8 was not lethal to mice and the spleens became slightly hyperplastic, while the sterile filtrate of *P. multocida* LFB3 BHI broth culture was lethal and induced a reduction in spleen mass.

Discussion

The results indicate that only bacterium-free sonicated extracts or supernates of broth cultures are suitable for exact determination of the toxin production of *P. multocida* strains in the mouse lienotoxicity test. In lower dilutions the extracts of toxigenic strains were lethal to mice and in mice surviving up to postinoculation day 7 they caused a reduction in spleen mass the degree of which was inversely related to that of dilution. The results obtained in this way showed good agreement with those of the guinea pig skin test aimed at demonstrating DNT production.

In the opinion of Éliás et al. (1986), after intravenous inoculation of *P. multocida* broth cultures the unchanged spleen mass of the surviving mice indicates toxigenicity, while the marked increase in spleen mass implies the lack of toxin production. Using the same method, in this study both toxigenic and non-toxigenic strains were found to cause an increase in spleen mass. There was no consistent correlation between lethality caused by the strains and toxin production either. This agrees with earlier observations made by other authors. Carter and Bain (1960) established that no relation between toxin production and virulence or infectivity for mice could be detected. By studying *P. multocida* strains isolated from rabbits and pigs Rimler and Brogden (1986) also found that the ability to produce toxin did not confer virulence to a serogroup D *P. multocida.*

Éliás et al. (1986) observed deaths among guinea pigs intradermally inoculated with BHI broth cultures of *P. multocida.* This may have been due to bacterial invasion, though they did not investigate this possibility. From the heart blood of guinea pigs that died after intradermal inoculation with live

cultures of *P. multocida* we successfully recovered large numbers of *P. multocida*. Studying the toxicity of *P. multocida* extracts in intraperitoneally inoculated mice, Rutter (1983) found that it was essential to remove viable bacteria by filtration. If this was not done, some non-lethal filtrates gave false positive results and large numbers of *P. multocida* were cultured from the peritoneal cavity of these mice at necropsy.

The results of the third experiment (Table III) confirm that, as opposed to *B. bronchiseptica*, *P. multocida* secretes its toxin into the culture fluid. The concentrated, sterile filtrate of *P. multocida* LFB3 broth culture was found to cause deaths among intravenously inoculated mice similarly to the cell-free sonicated extract. At a sublethal dose the filtrate resulted in atrophy of the spleen, indicating that it was also suitable for demonstrating the toxin in the lienotoxicity test. The question arises whether or not the two toxins are identical. Kamp et al. (1987) purified *P. multocida* DNT from bacterium-free broth culture fluid and it was found to be different from the toxin purified by Nakai et al. (1984) from a sonic extract of *P. multocida*. Further studies are needed to shed light on this problem.

It also calls for an explanation why viable *P. multocida* cells, which secrete toxin in the culture fluid, fail to cause atrophy of the spleen after intravenous inoculation. Contrarily, they induce an increase in spleen mass, while *B. bronchiseptica* cells not secreting toxin produce splenic atrophy in mice surviving intravenous inoculation with DNT-positive strains (Krüger and Horsch, 1982).

The results show that the mouse lienotoxicity test is suitable for studying the toxicity of *P. multocida* but only if cell-free extracts are used. The more toxic a strain, the higher dilutions of its extract are lethal and the more severe atrophy of the spleen develops in the surviving mice.

Finally, it should be emphasized that though the method presented here is suitable for demonstrating the toxicity of *P. multocida*, *in vitro* testing in cell cultures, where applicable, is a much more humane and economical procedure.

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INTERACTION OF T-2 FUSARIOTOXIN AND MONENSIN IN BROILER CHICKENS INFECTED WITH COCCIDIA

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Field observations suggest that coccidiosis is a common cause of death in broiler chicken flocks fed diets containing sufficient amounts of ionophore antibiotics (monensin, narasin, etc.) and contaminated with mycotoxins, particularly with T-2 fusariotoxin.

To study this phenomenon, broiler chickens fed diets containing different amounts of T-2 toxin and free from monensin, or containing a preventive dose (100 mg/kg of feed) of monensin, were infected experimentally with coccidian oocysts. In all groups fed a diet containing monensin plus T-2 toxin severe clinical symptoms of coccidiosis (blood-stained faeces etc). occurred. Deaths and retarded growth depended on the toxin dose and were considerable.

The body mass gain of chicks fed a diet containing monensin and T-2 toxin but not infected with coccidia was inferior to that of groups fed diets which contained either monensin or T-2 toxin (experiment 2). On the basis of these findings a negative interaction of the two compounds is assumed. This seems to be supported by the results of experiment 3, i. e. the finding that the lethal dose of narasin, a compound closely related to monensin both in chemical structure and mechanism of action, proved to be much lower (LD_{50} = 102 mg/kg body mass) for chickens fed a diet supplemented with T-2 toxin than for the control chickens (LD_{50} = 176 mg/kg body mass).

The present results suggest that the feeding of diets severely contaminated with T-2 toxin may alter the anticoccidial efficacy of monensin.

Keywords: T-2 fusariotoxin, monensin, coccidiosis

In spite of the wide use of highly effective anticoccidials (monensin, lasalocid, salinomycin, narasin, etc.), coccidiosis is rather common in broiler chickens. The most frequent cause of this is that the anticoccidial drug concentration of mixed feeds remains below the concentration required for preventive purposes. In many cases, however, the absence of anticoccidial efficacy could not be attributed to the lack, or insufficient quantity, of active substances. In a rather high proportion of these cases the feed was found to be contaminated with mycotoxins, especially with T-2 toxin. This finding prompted us to study the assumed relationship between mycotoxins and coccidiosis.

Mycotoxins may enhance the susceptibility of the organism to infections. An interaction was reported to exist between aflatoxin and caecal coccidiosis (Edds et al., 1973; Wyatt et al., 1975). Mortality due to caecal coccidiosis doubled in chickens treated with aflatoxin.

A pronounced interaction was established between ochratoxin A and coccidiosis, too (Huff and Ruff, 1981, 1982).

Recently, Hungarian authors (Sándor et al., 1988; Ványi and Sándor, 1988) have called attention to the interaction between deoxynivalenol and swine dysentery. Besides triggering and aggravating the disease, deoxynivalenol renders the otherwise effective treatment unsuccessful.

The objective of this experiment was to study the influence of T-2 toxin on the course of caecal coccidiosis; more closely, to determine whether or not it aggravates the disease and reduces the efficacy of preventive anticoccidial treatment.

Materials and methods

Inoculum. The caecal contents of chickens that had died of caecal coccidiosis in different places were collected, suspended in a large volume of 2% potassium bichromate and poured into Petri-dishes to form a thin layer. The suspension was kept at room temperature for 3 days to allow all viable oocysts to sporulate. Subsequently, the suspension was centrifuged at a low rpm and the supernatant was discarded. The sediment of the last centrifugation was made up to 100 ml with tap-water and the oocyst count (per ml) of this suspension was determined by modified McMaster's method. Only sporulated oocysts were counted. Practically all oocysts belonged to the species *Eimeria tenella.*

Inoculation of chickens. At the age of 10 days, chickens of the experimental groups were inoculated individually with 2 ml of an oocyst suspension containing 1.7×10^5 oocysts, using a thin rubber tube fitted on a syringe. One day later the same chickens were infected in groups by filling their drinking cups with oocyst suspension instead of drinking water. The concentration of this suspension was adjusted to 6×10^4 oocysts per 100 ml of water.

Production of T-2 toxin. Fusarium tricinctum (sporotrichioides) strain 216, isolated by us from feed, was inoculated on slant Sabouraud agar and incubated at 26 °C for 7 days. Subsequently sterile saline was poured onto the culture, and a spore suspension was prepared by mild stirring. One hundred g of rice was placed in a wide-mouthed, 500 ml Erlenmeyer flask, then 100 ml of 10% glucose solution was added and the flask was sealed with a cotton plug. The mixture was subjected to fractional autoclaving at 120 °C for 30 min on two consecutive days. After cooling down, it was inoculated with the spore suspension.

The suspension was incubated for three weeks, in the first and third week at 20–22 °C and in the second week at 5–8 °C. Subsequently the culture was dried at 60 °C, ground to fine flour, and assayed for T-2 toxin content. This fine flour material was mixed to the chicken diet in the necessary quantity. The

final mixture was checked for T-2 toxin content by a capillary gas chromatographic method (Ványi et al., 1982). The T-2 toxin content of the four samples ranged between 5.87 and 6.0 mg/kg.

Experiment I. Eight groups, each containing 10 Hybro day-old chickens of both sexes, were formed. All chickens received an anticoccidial-free broiler starter diet up to 7 days old. Subsequently the diet of groups 1, 2, 5 and 6 was supplemented with monensin sodium (100 mg/kg of feed) and/or T-2 toxin (6 mg/kg of feed). Chickens of groups 1–4 were infected with oocysts on day 10 or 11 (Table I). All groups were kept under observation for 28 days and the chickens' body mass gain was monitored. The organs of chickens that died and those killed on postinfection (PI) days 7 and 14 were processed for histological examination. The caeca were examined microscopically to assess the severity of coccidiosis and to compare the ratio of various developmental stages of coccidia. The spleen, liver, bursa of Fabricius, caecal wall and femur were examined histologically.

Experiment II. Eight groups were used also in this experiment. Each group contained 25 birds. From day 6 up to the end of the experiment all chicks except those of groups 4, 5, 7 and 8 received anticoccidial (100 mg monensin/kg of feed) and/or T-2 toxin containing diets as shown in Table II. The feed of group 4 was supplemented with 30 mg/kg vitamin E and 0.15 mg/kg selenium (sodium selenite). Chicks of groups 1–6 were infected with coccidia as described for Experiment I. Group 8 served as untreated control. The period of observation lasted 28 days.

Experiment III. Sexed broiler breeder cockerel chicks were fed anticoccidial-free starter diet from day-old up to 3 weeks old. At that age two groups of 50 birds each were formed. Group A continued to receive the same diet as before, whereas the diet of group B was supplemented with 2.5 mg/kg T-2 toxin. On day 3 after the beginning of toxin feeding, 10 chicks of both groups received, through a tube, Monteban 100 premix corresponding to 60, 100, 140, 180 and 220 mg/kg body mass of narasin, respectively, to determine the LD_{50} value.

The number of deaths within 48 hours following the treatment was recorded. It should be mentioned that after the 48th hour no further deaths occurred, though clinically ill chicks showing recumbency, locomotor disturbances and hanging their wings were still seen.

The LD_{50} of narasin was calculated according to Deichmann and Le Blanc.

Results and discussion

The results are summarized in Tables I and II. In both experiments chickens of the nonmedicated groups infected with oocysts of *E. tenella* devel-

Table I

Study of coccidiosis in broiler chickens treated with T-2 toxin (Experiment I)

Group	No. of chickens	Treatments			Deaths	Scores of clinical symptoms	Mean body mass gain between day 7 and 28		Total yield of groups* %
		I	A	T			g	%	
I/1	10	+	+	+	2	+++	342	81	48.6
I/2	10	+	+	—	—	+	362	85.8	68.7
I/3	10	+	—	+	7	+++	235	55.6	11.1
I/4	10	+	—	—	3	++	360	85.3	42.6
I/5	10	—	+	+	—	—	395	93.6	93.6
I/6	10	—	+	—	—	—	423	100	100
I/7	10	—	—	+	—	—	430	102	102
I/8	10	—	—	—	—	—	422	100	100

I: infection with coccidian oocysts (230,000 oocysts/chicken); A: anticoccidial supplementation of the feed (100 mg monensin sodium/kg of feed); T: T-2 toxin feeding (6 mg T-2 toxin/kg of feed); * in % of the control

Table II

Study of coccidiosis in broiler chickens treated with T-2 toxin (Experiment II)

Group	No. of chickens	Treatments			Deaths	Scores of clinical symptoms	Mean body mass on day 28		Total yield+ %
		I	A	T			g	%	
II/1	25	+	+	1 mg/kg	—	+	557	97.6	97.6
II/2	25	+	+	2 mg/kg	1	+	506	88.6	85.1
II/3	25	+	+	4 mg/kg	3	++	501	87.7	77.2
II/4*	25	+	0	0	2	+	475	83.2	68.8
II/5	25	+	0	0	7	+++	503	88.1	63.4
II/6	25	+	+	0	—	+	554	97.0	97.0
II/7	25	0	0	1 mg/kg	—	—	502	87.9	87.9
II/8	25	0	0	0	—	—	571	100	100

* given 30 mg vitamin E/kg of feed and 0.15 mg Se/kg of feed; +in % of the control

oped caecal coccidiosis leading to 30 and 28% mortality, respectively. The severity of coccidiosis depended on the intensity of infection. Although signs indicative of coccidiosis (blood-stained faeces) occurred also in groups given preventive treatment (group I/2, group II/6), these soon disappeared and did not lead to deaths.

In infected chickens whose diet contained both anticoccidial and T-2 toxin (groups I/1, II/1, II/2, II/3), severe caecal coccidiosis developed. In all groups but group II/1 varying numbers of deaths also occurred. There was a close correlation between the severity of clinical symptoms and the T-2 toxin

content of the diet. Coccidiosis was the most severe in the infected group fed a diet containing 6 mg/kg toxin (group I/3). In that group 70% of the birds died and the survivors also became seriously ill. This group was followed by the group fed 4 mg/kg and that fed 2 mg/kg toxin in the feed. In chickens fed 1 mg toxin/kg of feed no deaths occurred in spite of the clinical symptoms observed.

In the noninfected groups the toxin doses fed either alone or in combination with anticoccidial caused neither disease nor deaths.

There were significant differences in body mass gain. Groups infected with coccidia and fed toxin showed substantial retardation in body mass gain. The reduced gain found in some experimental groups was due partly to the deaths and partly to the small average body mass of the survivors.

Although in groups infected with oocysts under anticoccidial protection and not fed toxin no deaths occurred, their body mass gain was somewhat inferior to that of the control groups.

Microscopic examination of the caecal contents and mucosal scrapings clearly confirmed the clinical finding that coccidiosis was most severe in the toxin-treated groups.

Similar findings were obtained by microscopy of the cadavers' caecum. In the toxin-treated group the full width of the intestinal mucosa was necrotic, while in chickens not fed toxin necrosis was limited to the epithelium and upper third of the lamina propria (this was true both for the cadavers and the chickens killed during the experiment). In chickens not fed toxin the intestinal wall was often infiltrated with various inflammatory cells, and demarcation and repair with angiofibroblast tissue were also seen. No appreciable histological change occurred in the liver. Histological examination of the femur revealed, that the characteristics and thickness of the various ossification zones were similar in the different experimental groups. The histological picture of the spleen was also normal and corresponded to the birds' age. On the other hand histological examination of the bursa of Fabricius revealed differences between the groups. In the bursa of Fabricius of chickens killed in the toxin-treated groups there was moderate lymphocyte depletion, with necrosis of 5 to 10% of the lymphocytes. This was accompanied by mild interstitial fibrosis. The lesions were more expressed on PI day 7 than on PI day 14. Lymphocyte depletion, which is indicative of T-2 toxin action, was correlated neither with coccidial infection nor with the severity of coccidiosis.

Although the experimental design and the testing methods used did not permit an unambiguous determination of the mechanism by which T-2 toxin influenced the severity of coccidiosis, the results allow us to assume that T-2 toxin exerts this influence partly by its direct effect on the intestinal mucosa and partly by impairing immunological responsiveness, first of all the local immune response. T-2 toxin is known to decrease serum vitamin E concentra-

tion in chickens (Coffin and Combs, 1981). Possibly the reduced activity of the defence mechanism (primarily nonspecific) is also responsible for the more severe course of infection.

Moderate disturbances of blood coagulation may markedly aggravate the clinical picture of coccidiosis. Though haemorrhages are hardly characteristic of T-2 toxicosis, under field conditions T-2 toxin contamination of the feeds is often accompanied by the presence of other fusariotoxins (e.g. DAS) which typically cause haemorrhages diffusely but mainly under the digestive tract mucosa.

It was interesting to note that groups fed monensin and T-2 toxin simultaneously developed less quickly than did the controls, despite the fact that neither monensin nor T-2 toxin possessed a growth-depressing effect when given alone. The two compounds seem to have a negative interaction also in this respect. The existence of such a relationship was confirmed by the results of experiment III. The LD_{50} of narasin was found to be 176 mg/kg body mass. Feeding chickens on the same diet but containing also T-2 toxin substantially lowered the LD_{50} (to 102 mg/kg body mass). Since both compounds are primarily metabolized in the liver, the enhanced toxicity of narasin can probably be attributed to T-2 toxin induced functional damage of the liver parenchyma.

Vitamin E and selenium quantities exceeding the requirements of chickens reduce the severity of coccidiosis and enhance the birds' immune response to different antigens. From results obtained for group 4 of experiment II it appears that abundant vitamin E and selenium supply reduced the severity of clinical symptoms, decreased the mortality rate, and increased the total yield, in agreement with the findings of Colnago et al. (1984). The few stunted but surviving chickens, however, somewhat lowered the average body mass of that group.

The results obtained in experiments I and II seem to confirm our practical observation that consumption of feeds containing T-2 toxin aggravates the course of coccidial infection. In severe coccidial infection the ingestion of T-2 toxin may break through the protection conferred by anticoccidials (see experiment II), giving the semblance of resistance to the given compound (monensin).

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LIPID METABOLISM AND LOW MOLECULAR WEIGHT SOLUTE UPTAKE IN *ASCARIDIA GALLI*

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Adult *Ascaridia galli*, an intestinal nematode parasite of fowl, reveals a large variety of complex lipids such as phospholipids containing choline, ethanolamine, inositol, serine and glycerol. Lysophospholipid species, vinyl ether phospholipid (plasmalogen), neutral acylglycerols, cholesterol and non-esterified fatty acids are also present. Sugar-containing lipids, such as cerebrosides, sulphatides and gangliosides are abundantly present. Female parasites contain more lipids, particularly acylglycerols and phospholipids. Acylglycerols, phosphatidyl choline, phosphatidyl ethanolamine and glycolipids incorporate a large amount of radiolabelled precursor substrate in *A. galli*. The presence of important enzymes of lipid biosynthesis like glucose-6-phosphate dehydrogenase, malate dehydrogenase and hydroxymethyl glutaryl-CoA reductase as well as an enzyme of lipid ester hydrolysis, triacylglycerol lipase is detected in the parasite. These enzymes show subcellular distribution patterns and Michaelis–Menten kinetic characteristics comparable with that from rat liver homogenate. Studies on the uptake of labelled precursor molecules for lipid biosynthesis, glucose, acetate and palmitate show that the parasites can take up the isotopes readily in a time-dependent manner, showing substrate saturation kinetics, dependence upon Na ions, and can be inhibited by the presence of the bile salts sodium cholate and sodium deoxycholate. The substrate affinity constant (K_t) and maximum apparent velocity of glucose uptake in *A. galli* were found to be 9.09 mM and 26.67 mM per 100 mg tissue dry weight per min at 37 °C.

Keywords: *Ascaridia galli*, lipid composition, lipid biosynthesis, solute uptake

Ascaridia galli (Schrank) is a pathogenic nematode occurring in the intestine of domestic fowl (Sadun, 1950). It causes haemorrhage, enteritis and severe anaemia and results in considerable economic losses because of lowered egg and poultry production. Although carbohydrates are the major energy reserve in nematodes (Barrett, 1981), the role of lipids cannot be overlooked. Lipids are incorporated into eggs and are important constituents of membranes. They undergo turnover constantly. No report is available on the detailed lipid composition and lipid biosynthesis from simple precursors in *A. galli*. In this paper, we provide evidence that *A. galli* contains a large number of complex lipids and is capable of incorporating radiocarbon into the lipid fractions from ^{14}C-acetate. Also, an extremely active mediated process is present for the uptake of glucose, palmitate and acetate.

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Materials and methods

Materials. Adult *A. galli* were collected from the intestine of fowl procured from local poultry farms. The worms were thoroughly washed in saline in order to remove intestinal debris and adhering materials.

Chemical analysis. Lipids were extracted in chloroform: methanol (2 : 1 v/v) following the methods of Folch et al. (1957). Ganglioside-sialic acid was estimated in the lipid extract by the thiobarbituric acid method. Individual lipid classes of neutral (NL), phospho- (PL) and glycosphingolipid (GL) were separated by unidimensional chromatography on thin layer silica gel G plates as described by Sanyal et al. (1986). Various lipid components were identified by using specific spray reagents and cochromatography with authentic lipid standards (Supelco Inc.). Lipid spots were scraped off the plates and eluted from the silica gel by repeated extraction with a suitable solvent system prior to quantitation by chromogenic methods.

Total lipids were quantitated by the phosphovanillin method of Frings and Dunn (1970). Phospholipids (PL) were measured by phosphorus analysis after digestion with $HClO_4$ by the method of Bartlett (1959). Cholesterols (Chol) were quantitated by the ferric chloride–glacial acetic acid method of Zak (1957). Non-esterified fatty acids (NEFA) were estimated by the method of Lowry and Tinsley (1976). Cerebrosides and sulfatides were quantitated as their galactose moiety by the method of Svennerholm (1956). Plasmalogens were determined by the iodine absorption method of Williams et al. (1962). Acylglycerols were quantitated by glycerol analysis according to the method of Van Handel and Zilversmit (1957).

Dynamics of lipid metabolism. Incorporation of (Na)-1-^{14}C acetate into the lipid classes was studied in aerobic atmosphere in Krebs-Ringer bicarbonate buffer, pH 6.6, containing 1.0 g glucose in a volume of 1 litre. 95% O_2–5% CO_2 gas was passed through the buffer. Batches of female and male *A. galli* worms, weighing 100 to 150 mg, were incubated in Erlenmeyer flasks in a volume of 5 ml buffer containing 2.27 μCi of (Na)-1-^{14}C acetate (Bhaba Atomic Research Centre, Bombay, India) with 60.3 mCi/mmol specific activity in a Dubnoff-type metabolic shaker at 37 °C at 110 oscillations per min (to avoid the effects of unstirred water layers on uptake). Incubations were terminated at the indicated time intervals by adding large volumes of chilled buffer. The incubation medium was poured out of the flasks, the worms were washed several times with the buffer and gently blotted over a filter paper before further processing. There was no change in the motility of the worms at the end of the incubation period.

Lipids were extracted and fractionated as above. After removing I_2, the spots of individual lipid classes were scraped off directly from the developed chromatograms, suspended in a small volume of water and counted for radio-

activity using dioxane-based scintillation fluid (Bray's fluid; Butler, 1961). The liquid scintillation counter revealed more than 90% efficiency for ^{14}C counting (Rack Beta Liquid Scintillation Spectrometer, LKB Instruments).

Enzyme assays. The worms' ability to biosynthesize lipids was also tested by assaying the lipogenic enzymes, glucose-6-phosphate dehydrogenase (G-6-PDH) (Julian and Reithal, 1975), malate dehydrogenase (MDH) and hydroxymethyl-glutaryl (HMG)-CoA reductase (Rao and Ramakrishnan, 1975). A 10% homogenate of the parasites was centrifuged at $2000 \times g$ for 30 min at 4 °C and the supernatant served as the source of enzyme activity. G-6-PDH and MDH were assayed as a measure of change in OD at 340 nm of the reduction of NADP and oxidation of NADH, respectively, in a spectrophotometer-recorder (Uvikon 860, Kontron Instruments), using the kinetic mode.

HMG-CoA reductase was assayed in saline–arsenate homogenate, pH 5.5, with hydroxylamine hydrochloride as substrate, with and without adding 4.5 M NaOH. The ability of the worms to hydrolyse lipid esters was also tested by assaying triacylglycerol (Tg) lipase activity in parasite homogenates. The released fatty acid was estimated in Dole's reagent (Goss and Lein, 1967). Enzyme protein was quantitated by a modified sodium dodecyl sulphate–Lowry procedure (Lee and Paxman, 1972).

Michaelis–Menten kinetic parameters, V_{max} (maximum of apparent initial enzyme velocity) and Km (substrate affinity constant) of the parasitic enzymes were determined and compared with rat liver homogenates by assaying the enzyme at different substrate concentrations and constructing a Lineweaver–Burk double reciprocal plot of substrate and enzyme velocity from there of (Lineweaver and Burk, 1934).

Subcellular fractionation. A procedure of differential centrifugation was adopted for subcellular fractionation of the parasites in a buffer containing 0.25 M sucrose, 25 mM Tris-HCl, pH 7.3, and 1 mM ethylenediamine tetraacetate (EDTA), at 4 °C. The parasite homogenate was briefly sonicated and centrifuged at 3000 *g* for 15 min, then at 10,000 *g* for 30 min to obtain P_1 and P_2 fractions, respectively. 10 mM solid $CaCl_2$ was added to the supernatant in cold, centrifuged at 27,000 *g* for 30 min and the pellet (P_3) and supernatant (S) were collected. Fractions P_1, P_2, P_3 and S referred to nuclei plus debris, mitochondrial, microsomal and cytosolic fractions, respectively. All fractions were assayed for enzyme activity in the presence of 0.1% Triton X-100 to ensure complete lysis of organelles.

Determination of labelled substrate uptake. For radioisotope uptake studies, adult worms were incubated in 4 ml of Krebs-Ringer saline, containing 25 mM Tris-(hydroxymethyl) aminomethane-HCl buffer at pH 7.4 (KRT of Read et al., 1963), in the presence of 0.05 μCi of the designated radioactive compounds. Maleate was omitted from the buffer system of Read et al. as this compound

has been reported to have inhibitory effect on uptake (Welh, 1966). Worms recovered from the intestines of fowl were rinsed in KRT twice and randomised by host into groups of 2–3, each group constituting a sample. After incubation for 2 min at 37 °C in a shaking waterbath, the incubation medium was rapidly removed by aspiration. The parasites were washed in at least three 10 ml changes with ice-cold KRT and extracted in 2 ml of 70% ethanol for 48 h. Aliquots of the ethanol extract were counted for radioactivity by liquid scintillation spectrometry in a Beckman instrument using 4 g PPO, 50 mg POPOP + + 700 ml toluene + 300 ml methanol. The dry weights of ethanol-extracted worms were obtained from samples heated in tared foil pans for 24 h at 70 °C. To determine the effect of omitting Na^+ on glucose uptake, the Na^+ concentration of KRT was altered by replacing NaCl isosmotically with choline chloride, LiCl or KCl. Effects of bile salts on glucose and palmitate uptake were seen by adding the bile salts to the incubation medium. Radioactive substances used in this work (U-^{14}C-glucose, sp. act. = 160 mCi/mmol; (Na)-1-^{14}C acetate, sp. act. = 60.3 mCi/mmol; and (Na)-1-^{14}C palmitate, sp. act. = 23.3 mCi/mmol) were obtained from Bhabha Atomic Research Centre, Bombay, India. Data on the uptake of hexose as a function of substrate concentration were examined using plots of each of 3 linear transformations of the Michaelis–Menten equation: the 1/v versus 1/S of Lineweaver and Burk, the V versus V/S plot of Eadie, and the S/V versus S plot of Woolf. The most suitable is Eadie's plot since this has been found to be least sensitive to systematic deviation from Michaelis–Menten kinetics (Starling and Fisher, 1975).

Results

The quantitative analysis of lipid classes of adult male and female *A. galli* and the incorporation of ^{14}C-acetate are summarized in Tables I–IV. Female worms show a larger amount of total lipid while the radiolabel incorporation is greater in their male counterparts (Table I). The difference in total lipid content has been contributed by classes NL and PL. Males have more GL than females. PL comprise 55% of the total lipid of these worms, followed by 35% NL and 10% GL. However, the incorporation of label is greater in NL. The amount of total polar lipids and the polar/nonpolar ratio are slightly higher in males, while the PL/cholesterol ratio is almost the same in both sexes. Table II shows the individual PL compositions, of which phosphatidyl choline (PC) constitutes the highest amount while the other major PL are phosphatidyl ethanolamine (PE), phosphatidyl serine + phosphatidyl inositol (PS + PI) and sphingomyelin (SM), in decreasing order.

The vinyl ether aldehydogenic PL, plasmalogen (choline + ethanolamine combined) is also present in substantial amount in both sexes. Other minor

Table I

Lipid composition and incorporation of (Na)-1-^{14}C acetate into the lipids of adult *A. galli*

Lipid class	Male/Female	Lipid content μg/100 mg tissue wet wt	Incorporation of (Na)-1-^{14}C acetate into the lipid classes	
			At 90 min	At 180 min
			% of total lipid	
Total lipid	Male	9185.04 ± 10.05	—	—
	Female	9390.22 ± 12.07	—	—
Total phospholipid (PL)	Male	4604.05 ± 7.5	28.19	33.86
	Female	5174.75 ± 8.5	24.65	25.92
Total neutral lipid (NL)	Male	3184.57 ± 11.5	59.80	52.70
	Female	3501.88 ± 2.5	63.40	58.40
Total glycolipid (GL)	Male	1231.42 ± 3.5	11.99	13.40
	Female	534.84 ± 11.5	11.80	15.60
Total polar lipid (PL + GL)	Male	5835.47 ± 11.52	40.18	47.27
	Female	5709.59 ± 8.9	36.52	41.55
Polar/nonpolar	Male	1.832	0.6717	0.8964
	Female	0.613	0.5754	0.7110
PL/cholesterol	Male	7.567	2.3261	1.9230
	Female	7.832	1.16	1.3289

Results are expressed as mean ± SD of 3–4 observations. Incorporation of ^{14}C-acetate into total lipid of male and female *A. galli* amounted to 18,978 and 11,674 CPM/100 mg tissue wet wt, respectively.

Table II

Individual phospholipid composition and incorporation of (Na)-1-^{14}C acetate into the phospholipids of *A. galli*

Phospholipid class	Male/Female	Lipid content μg/100 mg tissue wet wt	Incorporation of (Na)-1-^{14}C acetate into the phospholipid classes	
			At 90 min	At 180 min
			% of total lipid	
Phosphatidyl serine + inositol	Male	1028.75 ± 7.25	6.58	7.77
	Female	1123.75 ± 8.33	6.16	6.37
Sphingomyelin	Male	1000.30 ± 2.34	6.85	8.24
	Female	1030.30 ± 3.33	5.73	5.73
Phosphatidyl choline	Male	1525.00 ± 10.07	7.24	8.40
	Female	1639.30 ± 9.08	6.37	6.58
Phosphatidyl ethanolamine	Male	1050.00 ± 7.77	7.50	9.42
	Female	1381.70 ± 8.33	6.37	7.22
Plasmalogen (Choline + ethanolamine)	Male	165.00 ± 1.25	—	—
	Female	178.75 ± 1.90	—	—

Results are expressed as mean ± SD of 3–4 observations

Table III

Individual neutral lipid composition and incorporation of (Na)-1-^{14}C-acetate into the neutral lipids of *A. galli*

Neutral lipid class	Male/Female	Lipid content μg/100 mg tissue wet wt	Incorporation of (Na)-1-^{14}C- acetate into the neutral lipid class	
			At 90 min	At 180 min
			% of total lipid	
Total acyl-glycerols	Male	936.93 ± 7.64	27.41	24.50
	Female	996.92 ± 6.66	27.34	27.60
Monoacyl-glycerols	Male	360.36 ± 5.75	8.56	7.85
	Female	237.38 ± 3.73	7.86	9.03
Diacyl-glycerols	Male	324.32 ± 4.73	6.33	6.40
	Female	154.30 ± 5.77	6.73	7.96
Triacyl-glycerols	Male	252.25 ± 2.55	12.51	10.10
	Female	605.34 ± 2.33	12.75	10.50
Total cholesterol	Male	608.30 ± 1 !.44	19.62	17.90
	Female	660.70 ± 13.67	21.25	19.60
Free cholesterol	Male	360.70 ± 7.95	10.40	9.11
	Female	360.70 ± 7.46	12.75	10.09
Esterified cholesterol	Male	247.60 ± 5.45	9.22	8.49
	Female	300.00 ± 6.20	8.50	9.56
Non-esterified fatty acid	Male	1639.34 ± 36.14	12.77	10.60
	Female	1844.26 ± 38.16	14.87	11.15

Results are expressed as mean ± SD of 3–4 observations

PL such as lyso PC (LPC), lyso PE (LPE), diphosphatidyl glycerol or cardiolipin (DPG), although detected, cannot be quantitated with confidence by phosphorus analysis and, therefore, are not reported. The presence of phosphatidic acid cannot be detected. There is no significant difference in biosynthesis patterns of these PL classes and no specific or differential incorporation into a particular PL species is noted.

The NL composition of *A. galli* is presented in Table III showing that acylglycerols and NEFA are the dominant NL classes of these nematodes. Female parasites contain slightly more triacylglycerols (Tg), NEFA and esterified fraction of cholesterol. However, incorporation studies show that radioactivity is almost evenly distributed among different NL species. Quantitative lipid analysis also reveals the presence of neutral GL, cerebrosides (glycosyl ceramides) as well as acidic GL such as gangliosides and sulphatides (sulphuric acid esters of cerebrosides). They are almost equally distributed in both sexes (Table IV). However, while cerebrosides and sulphatides are almost evenly distributed in terms of per cent of total lipid, gangliosides are present in extremely small quantity and incorporate small amounts of ^{14}C-acetate.

Table IV

Individual glycolipid composition and incorporation of (Na)-1-^{14}C-acetate into the glycolipids of *A. galli*

Glycolipid class	Male/Female	Lipid content μg/100 mg tissue wet wt	Incorporation of (Na)-1-^{14}C- acetate into the glycolipid classes	
			At 90 min	At 180 min
			% of total lipid	
Gangliosides	Male	59.08	1.73	3.22
	Female	66.32	1.48	3.18
Creebrosides (Neutral GL)	Male	575.92 ± 2.76	6.80	7.12
	Female	305.90 ± 1.37	6.03	7.76
Sulphatide	Male	655.50 ± 3.14	5.19	6.28
	Female	228.94 ± 1.03	5.83	7.86
Total acidic GL	Male	714.58	6.92	9.50
	Female	295.25	7.31	11.04
Neutral GL/acidic GL	Male	0.8509	0.9826	0.7494
	Female	1.036	0.8248	0.7028

Results are expressed as mean ± SD of 3–4 observations

Table V

Subcellular distribution of enzymes of lipid metabolism of *A. galli* and comparison of Michaelis–Menten kinetic parameters with that from rat liver

Enzymes (μmol/min/100 mg Protein)	V(μmol/100 mg protein/min)			
	Homogenate	Mitochondria	Microsome	Cytosol
G-6-PDH	3.85 ± 0.25	0.48 ± 0.02	0.417 ± 0.03	1.60 ± 0.09
MDH	34.83 ± 1.23	12.05 ± 0.73	6.28 ± 0.87	16.06 ± 1.01
HMG-CoA reductase	111.00 ± 0.98	184.00 ± 1.25	97.30 ± 0.79	160.00 ± 1.01
T_g-lipase	1.008	—	—	—

Enzymes (μmol/min/100 mg Protein)	Km(μM)		V_{max}(μmol/100 mg protein/min)	
	A. galli homogenate	Rat liver homogenate	*A. galli* homogenate	Rat liver homogenate
G-6-PDH	15.38	3.44	5.55	4.34
MDH	5.26×10^3	4.76	3.33	90.90
HMG-CoA reductase	0.5×10^6	4.08×10^3	133.33	501.72
T_g-lipase	3.33×10^3	—	47.63	—

Results are expressed as mean ± SD of 3–4 observations

The presence of lipogenic and lipolytic enzymes is detected in *A. galli*. G-6-PDH is more abundantly present in the parasitic cytosol than in mitochondria or microsomal compartments and, along with MDH and HMG-CoA reductase, shows lower substrate affinity (higher Km) as compared to the rat liver homogenate (Table V). HMG-CoA reductase and MDH are associated with mitochondrial fractions, although they are present in cytosol as well. The presence of Tg-lipase is also confirmed in the parasite homogenate.

The mechanisms of nutrient uptake from the environment by the parasite are characterized by incubation in a suitable assay system containing the radiolabelled isotopes of various low molecular weight compounds. Glucose, acetate and palmitate, which are known to be preferentially incorporated into the carbon skeleton of various lipid classes, are used and the radioactivity taken up by the parasite is measured in an ethanol-extracted fraction. The substrate uptake in *A. galli* has been found to be a non-linear, two-component process as a function of time and it also shows substrate saturation kinetics. An initial rapid uptake for the first two minutes is followed by a lower rate of uptake up to the 15th min when it reaches steady state (Fig. 1). From this study, experimental incubations of 2 min are chosen as optimal for further studies. Glucose uptake is also found to be sodium dependent and is consid-

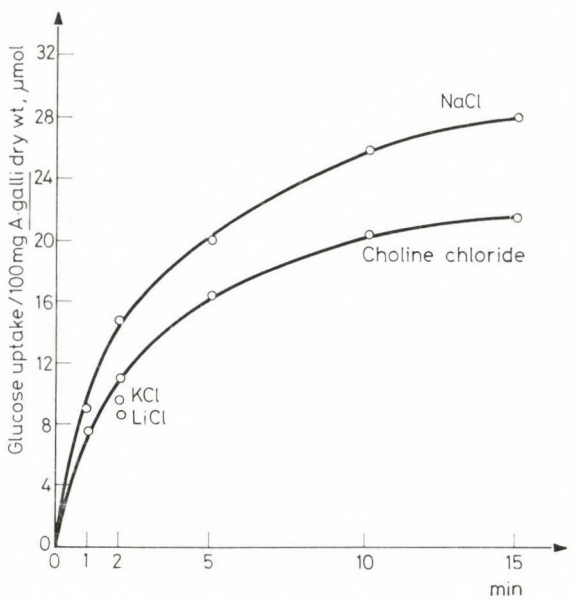

Fig. 1. Na^+-dependence of glucose uptake by *Ascaridia galli*. For details see Materials and methods. NaCl, choline chloride, KCl and LiCl were added to the standard incubation system in a concentration of 120 mM each. Values are the mean of three independent observations

erably lower in the presence of choline chloride, KCl or LiCl when these salts replace NaCl in the incubation medium. Also, glucose uptake is not linear with respect to substrate concentration, suggesting that the uptake is a mediated process, but no clear indication of a sigmoidal curve is evident (Fig. 2A). Linear transformation of the data on the effect of substrate concentration is also presented in the form of Eadie plot (Fig. 2B). The K_t and I_{max} of glucose uptake are 9.09 and 26.67 mmol · 100 mg tissue^{-1} · min^{-1}, respectively. Bile salts (Na cholate and Na desoxycholate), added to the incubation medium in a concentration of 10 mM, strongly inhibit the uptake of glucose and palmitate by the parasite (Table VI). The uptake of ^{14}C-acetate and ^{14}C-palmitate by the parasites also follows the same pattern as that of glucose (Fig. 3).

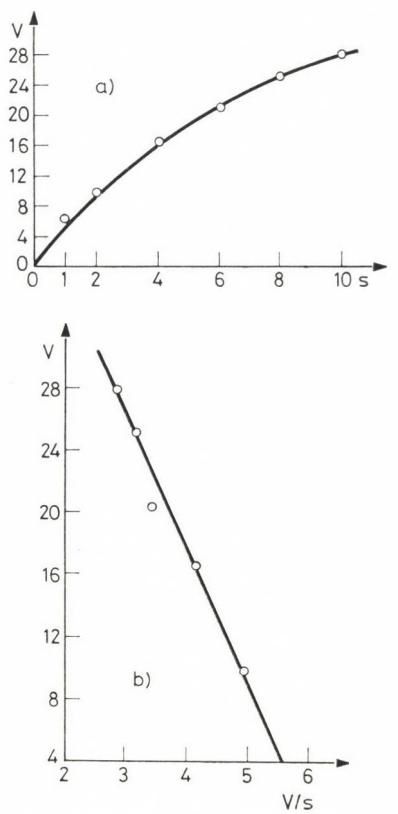

Fig. 2. Effect of substrate concentration on glucose uptake by *Ascaridia galli*. Uptake velocity (V) and substrate concentration (S) are expressed in mmol/100 mg of *A. galli* dry weight per min at 37 °C and mM, respectively. Values are the mean of three independent observations. (A) Uptake velocity versus substrate concentration curve. (B) Eadie plot of V versus V/S

Table VI

Inhibition of uptake of ^{14}C-glucose and ^{14}C-palmitic acid by *A. galli* during 2 min incubation with Na cholate and Na desoxycholate

Bile salt	Concentration mM	^{14}C-glucose uptake	^{14}C-palmitic acid uptake
		CPM/100 mg dry wt of *A. galli*	
Na cholate	0	9200 ± 141	15 474 ± 206
	5	6987 ± 648	12 656 ± 205
	10	4350 ± 189	7 434 ± 607
	20	5267 ± 1668	6 680 ± 63
	30	5751 ± 1541	5 494 ± 409
Na desoxycholate	0	9200 ± 141	15 474 ± 206
	5	5598 ± 1230	5 014 ± 215
	10	4834 ± 63	4 363 ± 60
	20	3823 ± 5	3 668 ± 81
	30	3760 ± 57	3 180 ± 47

Results are expressed as mean ± SD of 3–4 observations

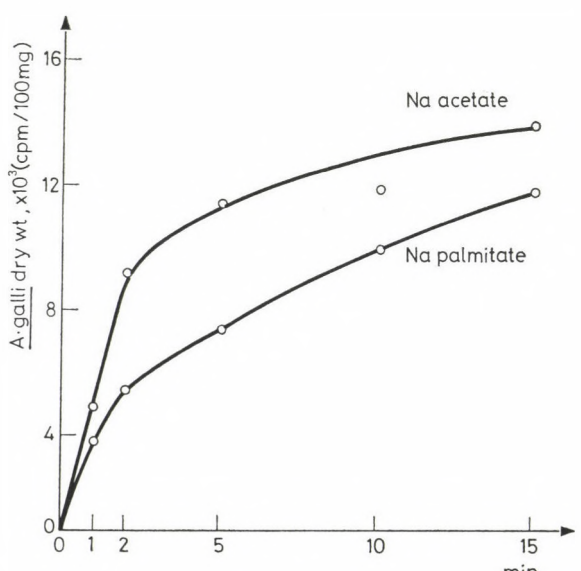

Fig. 3. Kinetics of Na acetate and Na palmitate uptake by *Ascaridia galli*. Values are the mean of three independent observations

Discussion

The results of the present study illustrate that adult male and female *A. galli* contain, and are able to biosynthesize *de novo*, all major classes of lipids including certain complex lipids. They contain plasmalogen (vinyl ether), a PL which may relate to the stability of ether bonds which cannot be hydrolysed by phospholipase. Therefore, it may contribute to the intactness of the parasite's surface membrane which has to survive in an unfavourable environment. Body wall lipids of *Ascaris* (Fairbairn, 1956) and also of the filaria *Litomosoides carinii* (Subrahmanyam, 1967) have been shown to contain plasmalogens. Cerebrosides and sulphatides are important GL and constitute the structural elements of the parasite's nervous system. These lipids, having a rather slow turnover, provide extreme stability to the nervous system and, therefore, may enable the parasites to ward off chemotherapeutic and other environmental assaults successfully. Gangliosides are present in extremely small quantity, predictably because they constitute the ligand molecules of the parasite's surface antigen only and are not a part of the structural organization. However, the much higher incorporation of labelled acetate into gangliosides is indicative of extremely fast biosynthesis and turnover, emphasizing their role as recognition molecules in the host–parasite interaction. They may also form part of the parasitic surface glycocalyx and are usually lost and reappear in the surface coat during moulting processes. The parasites also show a high PL/cholesterol ratio, which is the key determinant of membrane lipid fluidity, PL phase transition and membrane-associated integral protein functions, such as receptors, transporters and enzyme molecules (Shinitzy and Inbar, 1976).

Expectedly, most of the radioactivity incorporated into total lipids is found to be distributed in the PL, cholesterol, acylglycerol and fatty acid fractions, because the parasites have to meet the requirement of structural lipids for enormous egg production and rapid turnover of membranes. Tg is being synthesized more prominently than other acylglycerol species, while cholesterol is less esterified and, therefore, more available to be incorporated into the membranes. The high level of NEFA may provide the necessary stimulus for acylation of glycerol. NEFA may also form part of the parasitic secretion/excretion products besides being incorporated into the eggs. PC is the dominant PL species in these parasites. PC and PE may be important in maintaining continuity between the aqueous and lipid phases inside and outside the cell. Cardiolipin, which is mainly located in the inner mitochondrial membrane of higher organisms and has been characterized as the receptor for several mitochondrial enzymes including creatine phosphokinase (Müller et al., 1985), has also been detected in this parasite. In higher organisms most of the plasmalogen is also located in the mitochondria. Therefore, the presence of these PL,

which are usually associated with the skeletal muscles of higher organisms, may indicate that mitochondrial respirations and other functions are highly organized in this parasite, too. Lyso PL, LPC and LPE are probably breakdown products of the respective diacylated species. Their presence also suggests the probability of membrane-bound phospholipase enzyme activity in *A. galli*. The presence of phosphatidic acid could not be confirmed in our study although this PL has been demonstrated in the filaria *Setaria cervi* (Ansari et al., 1973).

The presence of lipogenic and lipolytic enzymes in *A. galli* and the kinetic characteristics comparable with those of higher animals may indicate that the parasite is not dependent on the host's lipid biosynthetic machinery and that the key enzymatic steps in intermediary metabolism are fully operative. G-6-PDH and MDH are important lipogenic enzymes providing the reducing equivalents, essentially needed for fatty acid synthesis, and also HMG-CoA reductase is the rate limiting enzyme in cholesterol biosynthesis. The presence of Tg-lipase in *A. galli* shows that the parasitic tissue is capable of hydrolysing acylglycerols. The presence of these enzymes assumes importance since in its usual habitat the parasite undergoes metabolism in anaerobic conditions or under stress of poor oxygen tension.

Adaptation of adult nematodes to life in the vertebrate gut requires dependence of the parasite on the supply of low molecular weight substances to be incorporated into the macromolecular structures. The uptake of glucose, acetate and palmitate by *A. galli* is accumulative, time dependent and a non-linear saturable function of external substrate concentration. Absorption, at least in part, is transcuticular (Wong and Fernando, 1970). Glucose uptake also seems to be a mediated system as in higher animals (Crane, 1965) and is also dependent on Na ions. Na^+ requirement for glucose influx is highly specific as it cannot be satisfied by choline, Li^+ or K^+. The uptake conforms Michaelis–Menten type substrate saturation kinetics as evidenced by a linear Eadie plot.

Glucose and palmitate uptakes were inhibited by bile salts, indicating that the success of the parasite requires integration with the host digestive system, as bile salts secreted by the host may perform a regulatory or limiting role in solute uptake. Na cholate and Na desoxycholate are known not to enter the parasite body (Surgan and Roberts, 1976) and, therefore, integration is a surface phenomenon. Probably, bile salt monomers adsorb to and saturate the loci specific for the transport of these solutes. Inhibition of uptake by bile salts is somewhat surprising, since it is well known that lipid uptake is increased from mixed micelles by bile salts in higher animals. Possibly the concentration of bile salts used in our experiments is high in the sense that, to ensure molecular contact between the solutes (fatty acid) and the absorptive loci, the bile salts must be present at their critical micellar concentrations. Increasing bile salt concentration above the critical micellar concentration may prove counter-productive.

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DNA RESTRICTION ENZYME ANALYSIS OF A BOVINE HERPESVIRUS 1 STRAIN ISOLATED FROM ENCEPHALITIS IN HUNGARY

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The DNA of a bovine herpesvirus 1 (BHV-1) strain isolated from calf encephalitis in Hungary was analysed with restriction enzymes. The cleavage pattern of the encephalitis strain Na/67 differed from those of all the other Hungarian BHV-1 isolates investigated so far. The *Eco*RI and *Hind*III cleavage patterns of virus strain Na/67 were found to be similar to the patterns of two other encephalitis strains (N569 and A663 from Australia and Argentina, respectively) characterized earlier. Strain Na/67 is the first isolate in Europe which showed the restriction enzyme pattern of BHV-1.3 previously supposed to be characteristic of encephalitis strains.

Keywords: Bovine herpesvirus 1, encephalitis, restriction enzyme analysis

BHV-1 may cause clinically, epizootiologically and pathologically different forms of disease in cattle. The virus was first isolated from animals suffering from a disease of the upper respiratory tract (infectious bovine rhinotracheitis, IBR; Madin et al., 1956). Later on the causative agent of IBR was found to be identical with that of infectious pustular vulvovaginitis (Kendrick et al., 1958). Subsequently, it was revealed that the virus can cause conjunctivitis (Abinanti and Plummer, 1961), encephalomyelitis (Johnston et al., 1962), abortion (Crane et al., 1964), and generalized systemic disease (Bartha et al., 1974). All these clinical entities caused by IBR virus have been observed in Hungary, too.

Although BHV-1 infection of cattle populations is widespread all over the world, the incidence of the diseases characterized by different symptoms is rather variable. While the respiratory form of the disease is permanently observed on all continents, encephalitis affecting young calves occurs sporadically. In the last three decades, BHV-1 associated encephalitis cases supported by isolation of the virus were reported in several countries.

The purpose of this study was to characterize the DNA of the Hungarian encephalitis strain designated Na/67 by means of restriction endonuclease analysis and to compare it with that of other known BHV-1 strains isolated from various clinical entities.

Materials and methods

Virus strains and virus propagation

The reference strains LA (Madin et al., 1956) and K22 (Kendrick et al., 1958) kindly supplied by Dr. H. Ludwig (West-Berlin) as well as the encephalitis strain Na/67 (isolated by Bartha et al., 1969) were propagated on monolayer cultures of secondary bovine testicle cells in roller bottles.

Virus purification and DNA extraction

Forty-eight h after infection, the culture medium was cleared from cells by low speed centrifugation. The virus from the supernatant was pelleted by centrifugation for 1.5 h at 25,000 rpm (SW 28 Beckman rotor). The pellet was resuspended in NTE buffer and centrifuged through sucrose cushion (30% w/v) for 1.5 h at 24,000 rpm. The pellet was resuspended in TE buffer and digested with Proteinase K (Sigma Chemical Company) at a concentration of 100 μg/ml in the presence of 0.5% sodium dodecyl sulphate. The viral DNA was purified by phenol–chloroform extraction and precipitated in 2.5-fold volume absolute ethanol with 0.3 M sodium acetate. The precipitate was pelleted, dried and redissolved in TE buffer. The methods used were described in more detail by Maniatis et al. (1982).

Restriction enzyme analysis

Restriction endonucleases were purchased from Amersham International plc, UK (*Eco*RI, *Hin*dIII) and from New England Biolabs, Beverly, MD, USA (*Bst*EII). Digestions were carried out under the conditions recommended by the suppliers. The DNA fragments were analysed by horizontal gel electrophoresis in 0.8% agarose according to Maniatis et al. (1982).

Results

Figure 1 shows the restriction enzyme patterns of the IBR reference strain LA, the IPV reference strain K22, and the encephalitis strain Na/67 obtained with the enzymes *Eco*RI, *Hin*dIII and *Bst*EII. It can be clearly seen that the restriction enzyme pattern of strain Na/67 is different from that of the IBR and IPV reference strains.

Since we were not in the possession of the encephalitis reference strains N569 or A663, their direct comparison with the Hungarian isolate Na/67 was not possible. Comparing the *Eco*RI and the *Hin*dIII restriction endonuclease

patterns of our strain with those of strains N569 and A663 published by Engels et al. (1986), the three encephalitis strains proved to be similar.

It is worth noting that the DNA restriction patterns of strain Na/67 differed also from those of all the other Hungarian BHV-1 virus isolates investigated so far (data not shown).

Discussion

Isolation of BHV-1 virus from calves with encephalitis has so far been reported in several countries: in Australia (French, 1962), the United States (Barenfus et al., 1963), Hungary (Bartha et al., 1969), Syria (Bagdadi and Martin, 1974), Scotland (Watt et al., 1981), Argentina (Carillo et al., 1983) and the Federal Republic of Germany (Meyer and Hübert, 1987).

Two out of the four strains associated with encephalitis which have been studied until now by restriction enzyme analysis, namely the North American (strain LAC) and the German (strain VI91) isolates, proved to possess DNA resembling that of the BHV-1 strains formerly called "respiratory" or "IBR-like" (Gregersen, 1983; Meyer and Hübert, 1987). These two strains could be classified as BHV-1.1 according to the labelling system suggested by Metzler et al. (1985). Because of the distinctiveness of their DNA restriction patterns (Brake and Studdert, 1985), the other two investigated isolates, i. e. the Australian strain N569 and the Argentinian strain A663, were grouped together as BHV-1.3 (Metzler et al., 1986). These two strains were identical when cleaved with *Eco*RI or *Hin*dIII, but could be differentiated with *Bst*EII (Engels et al., 1986).

The DNA restriction patterns of strain Na/67 generated by enzymes *Eco*RI or *Hin*dIII could not be distinguished from the published patterns of the above-mentioned two encephalitis strains. Using the *Bst*EII enzyme, strain Na/67 appeared slightly different from strain A663 and similar to strain N569. Strains Na/67 and N569 possessed an additional recognition site located at the map unit 0.420 compared to strain A663.

This is the first evidence of the occurrence of bovine encephalitis herpesvirus (BHV-1.3) infection in Europe. Since BHV-1.1 strains have also been reported as causative agents of calf encephalitis, it is supposed that there is no strict correlation between the clinical manifestation of BHV-1 infection and the genome type of the virus.

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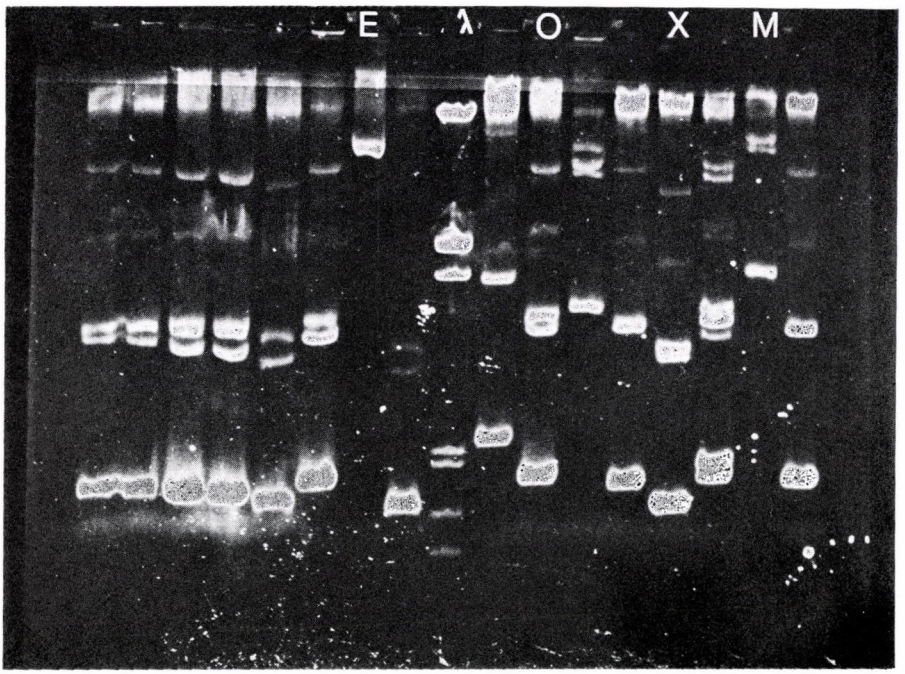

Fig. 1. Electrophoresis in 0.8% agarose gel of samples obtained by "mini" plasmid extraction to estimate the size of randomly cloned *Hin*dIII fragments. E = pUC18 containing *Hin*dIII fragment E; M = pUC18 containing *Hin*dIII fragment M; O = pUC18 containing *Hin*dIII fragment O; X = pUC18 corresponding in size to the original plasmid; λ = bacteriophage λ digested with restriction endonucleases *Hin*dIII and *Eco*RI (molecular weight standard)

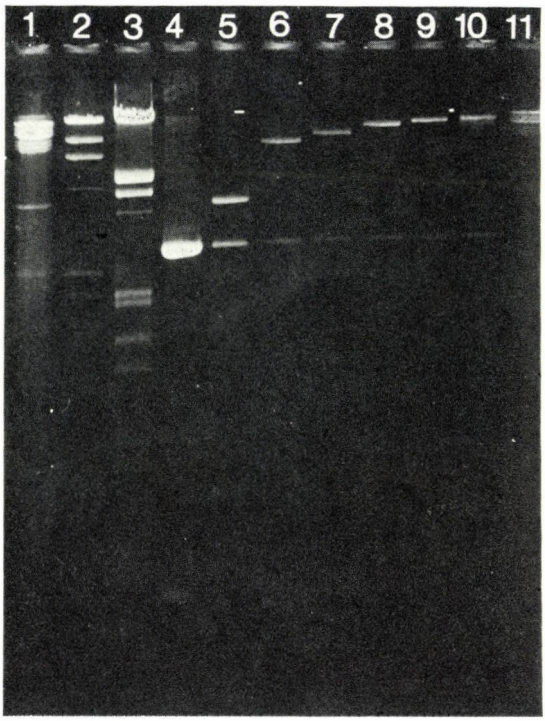

Fig. 2. Electrophoresis in 0.5% agarose gel of cloned *Hin*dIII fragments of the Hungarian IBRV isolate HB144. 1 = *Hin*dIII fragments of strain HB144; 2 = bacteriophage λ digested with restriction endonuclease *Hin*dIII; 3 = bacteriophage λ digested with restriction endonucleases *Hin*dIII and *Eco*RI; 4 = fragment O; 5 = fragment M; 6 = fragment L; 7 = fragment J; 8 = fragment G; 9 = fragment E; 10 = fragment D; 11 = *Hin*dIII fragments of the Hungarian IBRV isolate HB144

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MOLECULAR CLONING OF DNA FROM A BOVINE HERPESVIRUS 1 STRAIN ISOLATED IN HUNGARY

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Molecular cloning of the *Hin*dIII fragments of bovine herpesvirus 1 (BHV-1) strain HB144, isolated from infectious bovine rhinotracheitis (IBR) in Hungary, and of an infectious pustular vulvovaginitis (IPV) reference strain (K22) is reported. So far 52% of the IBR viral genome and 28% of the IPV viral genome have been cloned. The analysis of differences between the strains is currently in progress.

Keywords: Bovine herpesvirus 1, IBR, molecular cloning

Bovine herpesvirus 1 (BHV-1) is one of the most important herpesviruses pathogenic to cattle. It can cause a variety of syndromes (Ludwig, 1983) including respiratory disease (infectious bovine rhinotracheitis, IBR) of varying severity, conjunctivitis, abortion and, less commonly, it can be a cause of encephalitis in calves. This virus can be held responsible for causing infectious pustular vulvovaginitis (IPV) in cows and infectious balanoposthitis (IBP) in bulls.

Conjunctivitis, abortion and encephalitis caused by BHV-1 are frequently associated with the respiratory form. However, disregarding a few exceptional cases, the genital and respiratory forms do not occur together in the same herd (Kahrs and Smith, 1965).

These practical observations led researchers to assume that the different disease syndromes (mainly IBR and IPV) are caused by different virus strains. Initial studies revealed certain differences between the restriction endonuclease cleavage patterns of IBR and IPV isolates. The neuropathogenic strains recently reported from Australia and Argentina are distinctly different from the former (Engels et al., 1981; Engels et al., 1986; Ludwig, 1983; Seal et al., 1985). The next step was to classify the strains by their DNA structure and the clinical syndromes caused by them. Researchers hold divergent views in this respect. Some authors are of the opinion that the type of disease caused cannot be associated with differences found between the virus strains (Misra et al., 1983; Seal et al., 1985). No such relationship could be demonstrated by classical virological methods either (House, 1972). According to others (Engels et al., 1986), the three main groups established by analysis of the viral DNA include

three different types of strains, and strains within a given group produce the same clinical manifestation. Authors agree that there is very high (about 95%) genetic homology between IBR and IPV strains (Engels et al., 1986; Seal et al., 1985). With knowledge of this fact the diversified clinical manifestation of syndromes caused by BHV-1 is especially interesting. Other methods may also be useful in the analysis and classification of the strains: e. g. BHV-1 strains have been differentiated by their polypeptide patterns and selective reaction with monoclonal antibodies (Engels et al., 1986; Metzler et al., 1985; Pastoret et al., 1980).

No detailed study of viral DNA can be imagined without "having in hand" the genetic material of the given virus. It would, however, be lengthy and laboursome to grow the virus and purify the DNA again and again so as to make it available in sufficient quantity for a given work. Another problem is that the quality of DNA preparations produced at different times is inevitably different. This renders the interpretation of results rather difficult. Moreover, often there is no need for the whole DNA and only a certain region or selected fragment of the genome is needed for detailed study.

Molecular cloning of the viral genome affords a convenient solution to the problems outlined above. The molecular cloning of a Hungarian BHV-1 strain (HB144) isolated from respiratory disease (IBR) and of an IPV reference strain (K22) is reported in this paper.

Materials and methods

Virus propagation

Virus strains HB144 and K22 were grown in calf testicle cell cultures of low passage number and harvested at maximum cytopathic effect (48 h post-infection). Intracellular viral particles were released by three cycles of freezing and thawing.

Virus purification and DNA extraction

Cell debris was removed by centrifugation at 4000 rpm for 20 min. The virus-containing supernatant was centrifuged at 18,000 rpm for 150 min in a Beckman ultracentrifuge, with a type T-19 rotor. The virus pellet was resuspended in NTE buffer (100 mM NaCl, 10 mM Tris, pH 7.0, and 1 mM EDTA), layered onto 5 ml of 30% sucrose, and centrifuged at 24,000 rpm for 90 min in an SW-27 rotor. The virus pellet was again resuspended in NTE buffer (about 2 ml). 0.5 to 1.0% sodium dodecyl sulphate (SDS) was added to the sample and it was digested with 200 μg/ml Proteinase K (Sigma Chemical Co., St. Louis)

at 37 °C for 4 h. The DNA was extracted with a 25 : 24 : 1 mixture of phenol : : chloroform : isoamyl alcohol. Extraction was repeated 2 or 3 times. The purified DNA-containing solution was treated with a 24 : 1 mixture of chloroform and isoamyl alcohol. DNA was precipitated from the aqueous phase overnight at —20 °C by adding 10% 3 M sodium acetate and 2.5-fold volumes of dehydrated ethanol of —20 °C temperature. The precipitated DNA was centrifuged at 12,000 rpm for 30 min, the pellet was carefully washed with 70% ethanol, then centrifuged again as above. The supernatant was discarded, the pellet was dried briefly and, depending on the DNA quantity, resuspended in 100 to 300 μl of TE buffer (pH 7.9).

Molecular cloning

Plasmid and virus digestion. About 10 μg of DNA from the two viruses and about 5 μg of pUC18 plasmid used as vector (Yanisch-Perron et al., 1985) (Pharmacia Biotechnology International) were digested for 90 min at 37 °C with 12 units of the restriction endonuclease *Hin*dIII (Amersham International). Subsequently the DNAs were purified by phenol treatment and precipitation with ethanol. In the 60th min of pUC18 digestion, 22 U of alkaline phosphatase (Boehringer Mannheim GmbH) from calf intestine were also added to the reaction mixture.

Ligation. The ligation of the plasmid and the viral fragments was done in ligase buffer (10 μl 10x ligase buffer: 5 μl 1 M Tris-HCl, pH 7.6, 1 μl 1 M $MgCl_2$, 1 μl 1 M dithiothreitol, 3 μl 100 mM ATP) at 15 °C for 16 h with T4 DNA ligase enzyme (Biolabs, New England).

E. coli transformation. 200 ml of NM522 (supE, thi, Δ(lac-proAB), Δhsd5 (r-, m-), [F', proAB, $lacI^qZ\Delta M15$]) *Escherichia coli* culture (OD = 0.6 at 580 nm) was centrifuged at 3000 rpm for 10 min. The sediment was resuspended in 100 ml, then after another centrifugation (3000 rpm) in 20 ml of 0.1 M $CaCl_2$ solution and placed in ice for 20 min. Then it was centrifuged again as above and resuspended in 4 ml of 0.1 M $CaCl_2$ solution. The solution containing the ligated viral DNA fragments and plasmid was added to 100 μl of the bacterial suspension in a sterile Eppendorf tube and placed in ice for 20 min. Subsequently the mixture was placed in a 37 °C waterbath for 30 sec and kept in ice for another 30 sec. This was repeated 5 times (heat shock). Then the *E. coli* suspension transformed by the above procedure was inoculated into 1.2 ml 2×TY medium (1.6% bacto-tryptone, 1.0% yeast extract, 0.5% NaCl, 20 ml/l NaOH) and shaken at 37 °C for 30 to 45 min. First a mixture containing X-gal reagent (40 μl from 2% dimethylformamide solution of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and IPTG (100 μl from a 100 mM solution of isopropyl-β-D-thiogalactopyranoside), then 0.8 ml of the culture was spread with a bent glass rod onto agar medium containing 50 μg/ml

ampicillin [15 g of Bacto agar in 1 l of LB (1% bacto-peptone, 0.5% yeast extract, 0.5% NaCl, 10 ml/l NaOH)]. The cultures were kept in thermostat at 37 °C overnight. Of the white and blue bacterial colonies the white ones were inoculated into 5 ml of liquid LB medium containing 50 μg/ml ampicillin and shaken overnight at 37 °C. Subsequently the bacterial suspension was stored at 4 °C.

"*Mini*" *plasmid extraction.* 1.5 ml of bacterial culture was centrifuged for 30 sec (5000 rpm). The sediment was resuspended in 200 μl of STET (20% Triton-X 100, 0.2 M EDTA, pH 8.0, 1.0 M Tris, 20% saccharose) and, after adding 10 μl of 10 mg/ml lysozyme (Serva), boiled for 40 sec. Subsequently the samples were centrifuged at 13,000 rpm for 10 min. The supernatant was analysed by electrophoresis in 0.8% agarose gel to select clones containing plasmids of sufficiently increased size.

Plasmid production. 0.5 ml of each suspension containing the selected bacteria was inoculated into 200 ml of liquid LB medium containing 25 μg/ml ampicillin and the suspensions were shaken overnight. These bacterial suspensions were centrifuged at 3000 rpm for 10 min and the sediment was dissolved in 2.5 ml of 50 mM Tris buffer (pH 8.0) containing 25% saccharose. After adding 1 ml of 50 mM Tris buffer (pH 8.0) containing 10 mg/ml lysozyme the suspension was placed in ice for 5 min. Then 1 ml of 0.2 M EDTA solution (50 mM Tris, pH 8.0) was measured into the suspension, shaken, placed in ice for 5 to 10 min, then the whole suspension was transferred into SW-40 ultracentrifuge tubes. The tubes were filled up with 5.0 ml solution of 0.1 % Triton-X, 0.02 M EDTA and 50 mM Tris-HCl (pH 8.0) and centrifuged in a Beckman ultracentrifuge at 30,000 rpm for 45 min at 4 °C, without braking. 8.6 g of CsCl was measured into a TY 65 ultracentrifuge tube, and 8.6 ml from the supernatant of the centrifuged mixture and 86 μl of ethidium bromide were layered onto the CsCl. Subsequently the tubes were centrifuged in a Beckman ultracentrifuge (TY 60 fixed angle rotor, without braking) at 55,000 rpm for 20 h at 16 °C. The closed circular plasmid DNA labelled pink by ethidium bromide was collected by side puncturing the tube, with a sterile syringe, and was mixed with CsCl-saturated isopropanol. Using always the upper phase, the solution was purified by further four treatments with isopropanol saturated with CsCl and TE buffer (10.0 mM Tris, 1.0 mM EDTA). Finally the plasmid-containing solutions, from which ethidium bromide was removed by the above procedure, were dialysed for 5 to 15 h against TE buffer changed several times, then they were measured into Eppendorf tubes. The DNA preparation was stored at 4 °C (in the case of prolonged storage at —20 °C).

Analysis of the cloned fragments. To determine the size of DNA fragments inserted into the plasmids, the preparations were digested with the restriction endonuclease *Hin*dIII. Depending on the expected size of the fragments, the samples were analysed by electrophoresis in 0.5 to 1.4% agarose gel (together

with *Hin*dIII digested virus and molecular weight standards). For further identification of the clones the restriction endonucleases *Bam*HI, *Eco*RI, *Hpa*I (Amersham International) and *Bst*EII (Biolabs, New England) were used.

Results and discussion

The treatment of pUC18 vector cleaved with *Hin*dIII with alkaline phosphatase from calf intestine proved effective. As a result, the plasmids that had been cleaved by the enzyme could not reunite during ligation. Thus, only about 5% of the colonies were blue and all the rest (white colonies) contained a cloned fragment.

Certain authors recommend test digestion of samples obtained by mini plasmid extraction before selecting plasmids for preparation. In our experience, samples analysed without enzymatic digestion provide sufficient information on the size of the plasmids produced (Fig. 1).

Plasmids prepared for detailed study were examined to determine the DNA fragments that had been inserted by random cloning. Their size was determined by comparison with a molecular weight standard (bacteriophage λ digested with *Hin*dIII or with *Hin*dIII plus *Eco*RI). Subsequently we checked

Table I

Cloned *Hin*dIII fragments of BHV-1 strain HB144

*Hin*dIII fragments of IBR virus			Cloned	Contained by *Eco*RI or *Eco*RI-*Hin*dIII clone
Designation		Size (kilobase)		
1	A	21.2	—	—
2	B	18.95	—	+
3	C	16.35	—	partly
4	D	16.3	+	partly
5	E	13.75	+	partly
6	F	13.35	— (end-fragment)	+
7	G	12.25	+	—
8	H	12.25	— (end-fragment)	+
9	I	12.0	—	+
10	J	9.3	+	—
11	K	8.5	—	—
12	L	8.0	+	+
13	M	3.75	+	partly
14	N	2.4	— (end-fragment)	—
15	O	0.45	+	—

whether restriction enzymes *Bam*HI, *Bst*EII, *Eco*RI and *Hpa*I had cleavage sites in the given fragments and we determined the size of fragments produced by them. Comparing our results with physical maps available for the above enzymes (Engels et al., 1986; Mayfield et al., 1983), we determined the fragments contained by our clones.

About 90% of the inserted material is viral fragment and 5 to 10% cannot be identified. This latter part is thought to be DNA of tissue origin. The successfully cloned IBR virus fragments are listed in Table I. As random cloning of end-fragments of the viral genome cannot be expected, we can state that of the clonable fragments of IBR virus only fragments A, B, C, I and K have not been cloned yet (Fig. 2). The absence of fragments A and B is easy to understand, since these are the largest fragments with a size around the clonability limit. Cloning of fragments A and I by another method (direct cloning) is currently in progress. The *Eco*RI and *Eco*RI-*Hin*dIII clones derived from the same isolate, obtained by our team earlier, contain the viral regions represented by *Hin*dIII fragments B, F, H and I (Harrach, unpublished results). Thus, 52% of the viral DNA has been cloned in the form of *Hin*dIII fragments. Together with Harrach's above-mentioned clones, 74% of this isolate has been inserted into plasmid. Also the re-cloning of certain previously cloned parts of the genome is a result, as it has yielded smaller fragments easier to study.

Less has been achieved in the molecular cloning of the IPV isolate, strain K22 (*Hin*dIII E, H, J, M and P fragments, Fig. 3). However, here our aim only was to clone some interesting fragments for comparative studies, as this isolate had already been cloned (Engels et al., 1986; Mayfield et al., 1983). At present this work is continued by the direct method.

The physical map of the DNA of isolate HB144 molecularly cloned by us shows fairly good agreement with the published maps of the Cooper strain (Lawrence et al., 1986; Mayfield et al., 1983; Rock et al., 1987) and LA (Engels

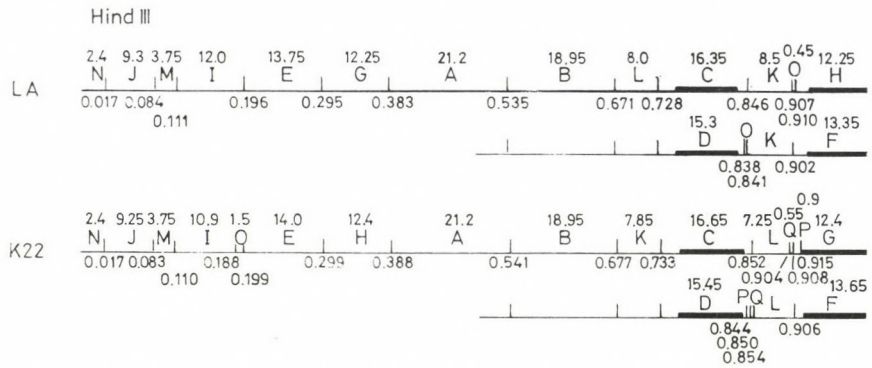

Fig. 3. Physical map of reference IBR (LA) and IPV (K22) virus strains (Engels et al., 1986)

et al., 1986) (Fig. 3). Identification and study of the minor differences are currently under way. However, from data of the literature and our experience it appears that so large deletions in the genome as those described for certain Aujeszky's disease (pseudorabies) virus isolates (Mettenleiter et al., 1988) cannot be expected in this case.

These results provide a basis for further genetic analysis of BHV-1 strain HB144. In addition, our clones may have concrete, directly utilizable practical significance. Perhaps they will prove more suitable for detecting IBR virus in nasal swab samples by hybridization than those few *Pst*I clones, randomly obtained from the same isolate, which are currently recommended for this purpose (Belák et al., 1988).

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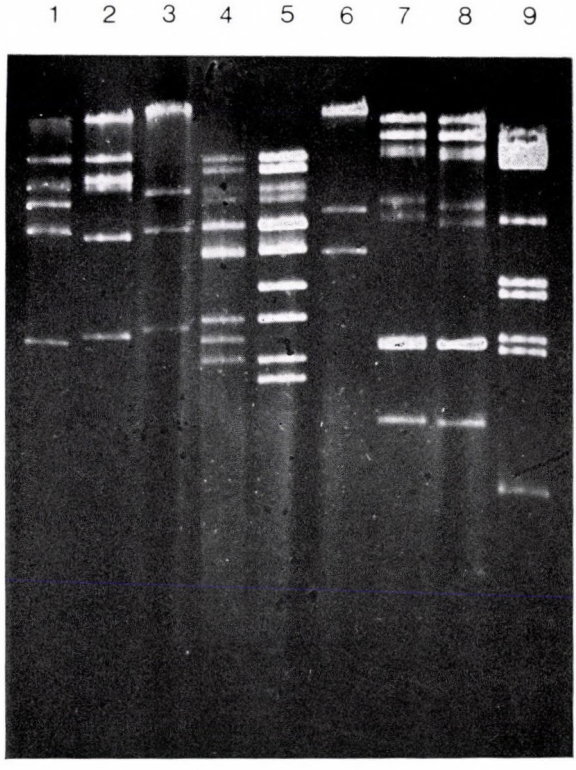

Fig. 1. Restriction endonuclease digests of BHV-1 strains cleaved with *Eco*RI (lanes 1–3), *Hin*dIII (lanes 4–6) and *Bst*EII (lanes 7–9). Lanes 1, 4 and 7: BHV-1.1 strain LA; lanes 2, 5 and 8: BHV-1.2 strain K22; lanes 3, 6 and 9: encephalitogenic Hungarian isolate Na/67

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THE CONSERVED OPEN READING FRAME OVERLAPPING THE THYMIDINE KINASE GENE OF DIFFERENT HERPESVIRUSES EXISTS ALSO IN BOVINE HERPESVIRUS 1

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Similar open reading frames (ORF) overlapping the thymidine kinase (TK) gene on the opposite strand or being close to it have been identified in 8 herpesviruses (Jacobson et al., 1989). Study of the DNA sequence of BHV-1 TK gene published by Mittal and Field (1989) and Kit and Kit (1986) revealed the existence of a similar ORF in this virus genome, too.

Keywords: Bovine herpesvirus 1, herpes simplex virus, thymidine kinase, overlapping ORF

Bovine herpesvirus 1 is responsible for considerable economical losses caused by abortion, respiratory disease (infectious bovine rhinotracheitis (IBR), infectious pustular vulvovaginitis (IPV), meningoencephalitis, etc. in cattle herds (Ludwig, 1983). The study of this virus is important also for comparative reasons as the genome organization of herpesviruses seems to be similar. A good example of these similarities is given here.

Part of an open reading frame (ORF) overlapping the thymidine kinase (TK) gene of herpes simplex virus type 1 (HSV-1) on the opposite strand in its 5' region (in a head-to-head orientation) was identified by Gompels and Minson (1986). The complete DNA sequence of this ORF was published by McGeoch et al. (1988), who designated it UL24. Studying the UL24, Jacobson et al. (1989) compared available DNA sequences from the respective regions of the genome of seven other herpesviruses and found similar ORFs.

The availability of the DNA sequence of the TK gene of bovine herpesvirus 1 (BHV-1) and its neighbouring genome region published by Kit and Kit (1986) and Mittal and Field (1989) made it possible to look for similar ORF in the respective region of this virus, too. The reverse complement strands of these DNA sequences were translated in three reading frames with the Pustell sequence analysis program version 4.0-IBM PC/XT-1983 (Pustell and Kafatos, 1984) run on an IBM compatible microcomputer (VARITER XT, MTA-SZTAKI COSY Engineering, Budapest).

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      1                                                           60
BHV-1                 MARDR DRRARLRAGI RCHSRFYEAL ACDA.RAAVG AQKLRPRLAQ
CMV                MPSGRGDDAD STGNALRRLP HVRKRIGKRK HLDIYRRLLR VFPSFVALNR
EBV                  MDPTRGLC ALSTHDLAKF HSLPPARKAA GKRAHLRCYS KLLSLKSWEQ
EHV                  MKRRQRL TARSRLRAGI RCHNRFYNA. MVQDLASAKR NGVYGERLAP
HSV-1              MAARTRSLV ERRRVLMAGV RSHTRFYKAL AEE..VREFH ATKICGTLLT
HSV-2 MARTGRRAAV GRPARTSSLT ERRRVLLAGV RSHTRFYKAF ARE..VREFN ATRICGTLLT
HVS                              MLS VIKQRDKEVL AHLPNKRKIA GNKAHLETYK
HVT                            MAGLLKAGV RGHNRFYKKL AAENFDQSSA GNTVGRTLQK
VZV                    MSASR IRAKCFRLGQ RCHTRFYDVL KKDIDNVRRGFADAFNPRLAK
CON                           RR  LRAG  R H RFYKAL A     R           L

      61                                                          120
BHV-1 LLGKFGAPEV FKQVVGVSLS FEVNLQSRRP DCVCLLRVAE AGHARA.... ..VCLIVELK
CMV   LLGG..LFPP ELQKYRRRLF IEVRLSRRIP DCVLVFLPPD SGSRG..... IVYCYVIEFK
EBV   LAS.FLSLPP GPTFTDFRLF FEVTLGRRIA DCVVVALQPY PRCYIVEFKT AMSNTANPQS
EHV   LFSELVPAET LKTALGVSLA FEVNLGQRRP DCVCTVQFGK GSDAK..... .GVCILIELK
HSV-1 LLSGSLQGRS VFEATRVTLI CEVDLGPRRP DCICVFEFAN D...KTL... GGVCVIIELK
HSV-2 LMSGSLQGRS LFEATRVTLI CEVDLGPRRP DCICVFEFAN D...KTL... GGVCVILELK
HVS   KLAKYTVSAS IFKFLSISHP CPLRAKTRLF FEVSLGNRIA DCVMLTSCGE TRICYVIELK
HVT   ILSKILPSEL LKGASAFRLA FEVDLGRRRP DCICMF...T LSGGLLVEGC NNVCVIIELK
VZV   LLS.PLSHVD VQRAVRISMS FEVNLGRRRP DCVCIIQTES SGAGKT.... ..VCFIVELK
Con   LLS  L        A RVSL  FEV LGRRRP DCVCVF         KT        VC IIELK

      121                                                         180
BHV-1 TC.....RFS TNMNTPSKMD QRLGGLRQLR DSARLVRDLA PP..GPDPVV LAPVQVFVSQ
CMV   TTYSDADDQS VRWH.ATHSL QYAEGLRQLK GALVDFDFLR LPRGGGQVWS VVPSLVFFQQ
EBV   VTRKAQRLEG TAQLCDCANF LRTSCPPVLG SQGLEVLAAL VFKNQRSLRT LQVEFPALGQ
EHV   TC.....RFS KNMNTASKNL QRKGGMRPVH DSCRLLARTL PP..GSGEIV LAPVLVFVAQ
HSV-1 TCKY.....I SSGDTASKRE QRATGMKQLR HSLKLLQSLA PP..GDKIVY LCPVLVFVAQ
HSV-2 TCK.....SI SSGDTASKRE QRTTGMKQLR HSLKLLQ
HVS   TCMTSNLDLI S....DIRKS QRSQGLCQLA DTVNFIHNYA PLGRQAWTVL PILIFKSQKT
HVT   TC.....RFY RNLKTASKNE QRATGTKQLL ESKTLIERMA P..RGSDHTI ICPVLVFVAR
VZV   SC.....RFS ANIHTPTKYH QFCEGMRQLR DTMALIKETT P..TGSDEIM VTPLLVFVSQ
Con   TC     RFS  N  TASK   QR  GMRQLR DS  L    A PP  G       L PVLVFV Q

      181                                                         240
BHV-1 RGMRVLRVTR LPAQTIASNA ARLEAIIAGL AEYAPFARAR SRRAGRSPRG KRKAEQPRPR
CMV   KADRPSFYRA FRSGRFDLCT DSVLDYLGRR QDESVAHLLA ATRRRLLRTA RGKRAALPRA
EBV   KTLPTSTTGL LNLLSRWQDG ALRARLDRPR PTAQGHRPRT HVGPKPSQLT ARVPRSARAG
EHV   RGMRVLRVTR LSPQVVYSNA AVLSCTISRL AEYAPPVSAK STRRRCV... AKGTKAKAFS
HSV-1 RTLRVSRVTR LVPQKVSGNI TAVVRML... .....QSLST YTVPIEPRTQ RARRRRGGAA
HVS   LKTLHIETPK FPVNLTHTSE EKLSCFLWSR ADVEIRKKIH LAPKPKRIFK WDSLLDSTST
HVT   RGLVVIRSTP L.KKKAICTD FHRLASMMYL RSEYRLYDSG MCKPMRKICH RNKRTVSTVR
VZV   RGLNLLQVTR LPPKVIHGNL VMLASHLENV AEYTPPIRSV RERRRLCKKK IHVCSLAKKR
Con   RGLRV RVTR L                L    A             R

      241                                                         300
BHV-1 RQKGQPLPLA TGKRAAVAAP RGRPPATPAL LRPGTAAAGG RPAGTRATAP AGALKTRRAA
CMV   RASAVAGGRG GDNARRGLAR GRAHGPGAQT VSASGAQGSG SQGADLLRGS RRARVRGGGA
EBV   RAGGRKGQVG AVGQVCPGAQ K
EHV   TKAAAEPVPS ITPAQPSAAA AVVSLFPAAV PANTTNAAAV HQPVAVSHVN PLAWAASLFS
HSV-1 RGSASRPKRS HSGARDPPES AARQLPPADQ TPTSTEGGGV LKRIAALFCV PVATKTKPRA
HVS   EHSAYRQKLI ERNKKKCFTL QNQTSKFRDR PNKKSNDQLR ARQANARPCK KKQHNNKRLR
HVT   RMRCGISDTG VLPIEKQHVD ARGKDCGTSG IEGEPIMQRM RNIISHLSRR H
VZV   AKSCHR.... .SALTKFEEN AACGVDLPLR RPSLGACGGI LQSITGMFSH G
Con   R SA                 A

      301                                                         360
BHV-1 RRAWAKFRRS LWRHRGHGAR AFRVCLPGRG HPRSRGPRLC TGRAKFYTPA FPLRFWDEPV
CMV   VEPAVRARRR TVAADAATTT VSSAFFVPRD RRGRSFCRPT RSL
EHV   PK
HSV-1 ASE
HVS   NNRKHGGKVS RLTTTTSFSS EAAFSNYPVS THKL
Con

      361                                                         420
BHV-1 FARSWGRGRA RAGAGREAAG AEPGRQRPRT TCWRRQTPRA RKSTRASGPL RPGGGGSRRA

      421                   447
BHV-1 RGPGGGGAPG GGGGPAAESA RARAAPP
```

The computer analysis showed an ORF on the opposite strand to the TK gene in a head-to-head orientation overlapping only in the two base pairs of the A and T of the start codons. This ORF showed striking similarities to the UL24 like ORFs of 8 other herpesviruses (Fig. 1). The consensus line shows 100 conserved amino acids present at a given position at least in four different viruses. The BHV-1 shares 79 of these conserved amino acids. The similarities both in the position (head-to-head overlapping on the opposite strand of the 5' end or being close to it; shared by 8 herpesviruses of the nine cited ones) and in the amino acid composition (especially in the regions strongly conserved in the other herpesviruses) suggests the conclusion that this BHV-1 ORF is equivalent to the other UL24 like ORFs.

The DNA sequence published by Mittal and Field (1989) covered the first 81 amino acids of this ORF. The sequence of Kit and Kit (1986) contains the whole ORF, with a length corresponding to 418 amino acids. There were some discrepancies between the published TK sequences of these two groups (Mittal and Field, 1989) but the sequences from the TK overlapping ORF were identical.

This newly identified ORF seems to be the longest among the other similar sequences available (Fig. 1). Its predicted amino acid sequence gave information to identify 25 further conserved amino acids to the ones found by Jacobson et al. (1989).

The effect of clustered point mutations within the UL24 of HSV-1 was studied by Jacobson et al. (1989), who concluded that this ORF is important for viral growth. Martin et al. (1989) showed a similar phenomenon in the herpesvirus of turkeys. They failed to recover viruses with inserts in the UL24 like ORF and found that viruses with inserts in the TK gene close to this ORF grew very poorly.

There is a growing interest in producing well characterized attenuated live BHV-1 vaccines for immunization against IBR. A genetic engineering solution was suggested to make deletions in the TK region of BHV-1 (Kit and Kit, 1986). However, in any such attempt or when evaluating gained mutants one should consider the possible negative effects of a deletion in or close to the hereby described UL24 like ORF of BHV-1.

Fig. 1. The predicted amino acid sequence of the TK overlapping ORF of bovine herpesvirus 1 (BHV-1; Kit and Kit, 1986; Mittal and Field, 1989) and its optimal alignment with 8 other herpesviruses: human cytomegalovirus (CMV; P. Tomlinson, C. M. Brown, A. T. Bankier and B. G. Barell, personal communication, cit. Jacobson et al., 1989), Epstein-Barr virus (EBV; Bankier et al., 1983), equine herpesvirus (EHV; G. Robertson, personal communication, cit. Jacobson et al., 1989), herpes simplex virus type 1 (HSV-1; Gompels and Minson, 1986; McGeoch et al., 1988), herpes simplex virus type 2 (HSV-2; Kit et al., 1983; Swain and Galloway, 1983), herpesvirus saimiri (HVS; U. Gompels, personal communication, cit. Jacobson et al., 1989), herpesvirus of turkeys (HVT; Martin et al., 1989), varicella zoster virus (VZV; Davison and Scott, 1986). Gaps were inserted (represented by dots) to achieve optimal alignment. The consensus line (Con) shows the amino acids present at the position at least four times

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AN EVALUATION OF FOUR SEROLOGICAL TESTS FOR THE DETECTION OF ANTIBODIES TO BOVINE PARAINFLUENZA VIRUS TYPE 3

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Haemagglutination-inhibition (HI), virus neutralization (VN), haemolysis-inhibition (HLI-1, HLI-2) tests, and two new haemofusion-inhibition (HFI-1, HFI-2) tests, developed by us, were used for the detection of antibodies to bovine parainfluenza virus type 3 (BPIV-3) in sera of 45 steers randomly selected from a herd naturally infected by BPIV-3. Twelve seronegative animals were then vaccinated with formalin-inactivated vaccine and 15 days later tested by the above-mentioned assays. Linear regression analysis revealed positive correlations between HI, VN, HLI-1 and HFI-1 antibody titres, which confirm the specificity of the HFI-1 test. The HLI-1 and HFI-1 assays proved to be less sensitive than the HI and VN ones, while HLI-2 and HFI-2 tests failed to detect any antibody to BPIV-3.

Keywords: Bovine parainfluenza virus type 3, serum antibody, serological tests

An important, but still unsolved, problem of bovine parainfluenza virus type 3 (BPIV-3) and other paramyxovirus infections is how to estimate the antibody response to the HN and F glycoproteins of the viral envelope separately. Results published by Rydbeck et al. (1986) and Shibuta et al. (1986) suggest that the HI assay can be used for studying the anti-HN antibody response. These authors, using monoclonal antibodies to parainfluenza 3 virus, found that only antibodies to some epitopes on HN glycoprotein, the antibodies preventing virus attachment to erythrocytes, possessed HI activity. On the other hand, one cannot tell what kinds of antibodies are estimated in the VN and HLI assays, since each of the corresponding monoclonal antibodies can express both VN and HLI activities (Rydbeck et al., 1986; Shibuta et al., 1986). The F glycoprotein, which is responsible for virus penetration into the cell (for a review, see Compans and Klenk, 1979), may be considered a candidate for paramyxovirus subunit vaccines to be developed against canine distemper (Norrby et al., 1986), mumps (Löve et al., 1986) and Newcastle disease (Meulemans et al., 1986) viruses. In the case of human parainfluenza 3 virus the presence of both anti-HN and anti-F antibodies has been found to be essential for protective immunity (Rydbeck, 1987; Ray et al., 1988).

The aim of the present study was to evaluate serological tests based on the inhibition by specific antibody of BPIV-3 biological activities associated with the BPIV-3 F glycoprotein, i. e. haemolysis and erythrocyte fusion (haemofusion) and to compare the results with those of the routine HI and VN assays.

Materials and methods

Virus. BPIV-3 strains SF-4 and Lublin-1, kindly provided by Prof. J. Buczek (Agricultural Academy, Lublin, Poland), were grown in Madin-Darby bovine kidney (MDBK) cells. Formalin-inactivated vaccine (Deptuła and Buczek, 1984) was obtained from Dr. W. Deptuła (University of Szczecin, Poland).

Serum samples were collected from 45, approximately one-year-old, Polish lowland black-and-white steers. The sera were heated at 56 °C for 30 min and, before tested in the HI, HLI and HFI assays, were kept at 37 °C for 90 min with an equal volume of 10% guinea pig red blood cells to absorb nonspecific antibodies.

The *haemagglutination-inhibition (HI) test* was carried out by the standard method (Rentschler, 1970); 1/5th volumes of components were used in the microtitre plates (Plastomed, Warsaw, Poland). Titres of 16 and higher were considered positive (Rentschler, 1970; Morein et al., 1973).

Virus neutralization (VN) assay. Two hundred $TCID_{50}$ of strain SF-4 in 50 μl of Eagle's minimum essential medium (MEM) was mixed with equal volumes of serial 2-fold dilutions of serum in MEM. The mixtures were incubated for 1 h at 37 °C. Subsequently, 30 μl volumes of each virus-serum mixture were transferred into three wells of 96-well type F plates (Plastomed, Warsaw, Poland) containing confluent monolayers of MDBK cells previously washed two times with PBS. Following an adsorption period of 1 h at 37 °C 150 μl of Parker's medium enriched with 1% fetal calf serum was added to each well. The results were read after an incubation for 5 days at 37 °C in a humidified CO_2 incubator. The highest serum dilution giving protection of cells in two of the three wells was taken as the VN titre (Fischman, 1967). Titres of 4 and higher were considered to be positive.

Haemolysis-inhibition 1 (HLI-1) test. A hundred μl volumes of strain SF-4 (about 64 haemagglutination units), frozen and thawed 8 times before, were added to 50 μl of 2-fold serum dilutions in PBS. The mixtures were kept at room temperature for 30 min, then the test tubes were transferred into an ice-bath, and 100 μl of 10% guinea pig erythrocytes were added. After an adsorption period of 30 min, the red blood cells were sedimented by centrifugation at $500 \times g$ for 5 min and the supernatants were removed. Subsequently, 1.5 ml of PBS-A (without calcium and magnesium ions), pH 7.0, containing

5 mM EDTA, was added to the sedimented cells. After an incubation for 1 h at 37 °C, with manual shaking from time to time, the unlysed erythrocytes were pelleted by centrifugation and the optical density of the supernatant was determined at 540 nm. The reciprocal of the final dilution of serum causing a 50% reduction of haemolysis was considered to be the HLI-1 titre. Titres of 8 and higher were taken as positive.

In the *HLI-2 test* equal volumes of virus antigen and erythrocyte suspension (components as used in the HLI-1 test) were mixed and kept in an ice-bath for adsorption for 30 min. Then 200 μl volumes of this virus-erythrocyte suspension were added to 50 μl of serial 2-fold dilutions of serum in PBS of 0 to 4 °C temperature. After shaken, the test tubes were kept in an ice-bath for 30 min, and the remaining procedure was the same as described for the HLI-1 test.

Haemofusion-inhibition 1 (HFI-1) test. Strain SF-4 previously subjected to four cycles of freezing and thawing was used as antigen. Its haemofusing activity was determined as described elsewhere (Trybała et al., 1989). The smallest amount of virus causing detectable fusion, i. e. 10 polyerythrocytes in 10 microscope fields (magnification: $\times$ 200) was considered one haemofusing unit (HFU). The HFI-1 test was carried out as follows: Four HFUs of virus antigen in 100 μl were added to 50 μl of serial 2-fold dilutions of serum in PBS. The mixture was kept at room temperature for 30 min, then transferred into an ice-bath, and 50 μl of a 0.3% guinea pig erythrocyte suspension was added. After 30 min of adsorption, the cells were sedimented at $450 \times g$ for 5 min and the supernatant was carefully removed. Subsequently, 100 μl of saline buffered with 0.02 M phosphate, pH 7.0, containing 2 mM $CaCl_2$ was added to the sediment and, after an incubation for 15 min at 37 °C, the test tubes were transferred into an ice-bath. After dispersion of the sediment with a micropipette, 35 μl of the suspension was placed on a slide and spread over a surface of about 1 cm^2. The results were read under the microscope at a magnification of $\times$200. The reciprocal of the highest serum dilution in which less than 10 polyerythrocytes were found in 10 microscope fields, was arbitrarily taken as the HFI-1 titre. Titres of 8 and higher were considered positive.

In the HFI-2 test erythrocyte suspension and virus antigen (the same components as those used in the HFI-1 test) were mixed at a ratio of 1 : 2 and, for adsorption, were kept in an ice-bath for 30 min. Subsequently, 150 μl of this virus-erythrocyte suspension was added to 50 μl of serial 2-fold dilutions of serum in PBS previously cooled to 0–4 °C. After shaking, the test tubes were kept in ice-bath for 30 min and the rest of the procedure was performed as described for the HFI-1 test.

Statistical evaluation of data was done by linear regression analysis.

Table I

HI, VN, HLI-1 and HFI-2 antibody titres in sera of steers

Steer no.	Serological test							
	HI		VN		HLI-1		HFI-1	
1	64		8		16		16	
2	128		16		16		16	
3	64		8		32		16	
4	16		8		8		4	
5	8	(128)	4	(128)	8	(32)	<4	(16)
6	64	(512)	32		16		16	
7	8	(512)	4	(64)	4	(128)	4	(128)
8	64		32		16		16	
9	8	(512)	16	(128)	4	(64)	<4	(64)
10	32		32		8		8	
11	16		8		4		8	
12	16		16		4		4	
13	16		32		8		4	
14	<4	(512)	<2	(256)	<4	(32)	<4	(32)
15	16		8		4		4	
16	8	(256)	8	(64)	4	(64)	4	(32)
17	<4	(64)	<2	(16)	4	(16)	<4	(16)
18	64		64		16		32	
19	8	(1024)	2	(256)	<4	(128)	8	(64)
20	32		16		8		8	
21	16		16		8		8	
22	16		8		4		8	
23	4	(512)	2	(128)	4	(64)	4	(64)
24	16		8		8		16	
25	16		4		8		16	
26	8	(512)	4	(512)	8	(128)	4	(128)
27	32		16		8		8	
28	32		16		8		8	
29	8	(1024)	2	(1024)	4	(256)	4	(128)
30	128		32		32		64	
31	16		16		4		8	
32	8	(64)	2	(16)	<4	(16)	4	(16)
33	8	(512)	2	(32)	4	(64)	8	(64)
34	64		32		16		8	
35	32		16		16		16	
36	32		4		8		16	
37	16		8		16		16	
38	64		16		8		16	
39	16		32		4		<4	
40	16		8		8		4	
41	64		32		32		8	
42	64		16		8		8	
43	32		16		16		8	
44	32		8		4		8	
45	16		16		4		8	

In parentheses: titres 15 days after vaccination

Results

The HI, VN, HLI-1 and HFI-1 titres found in sera of 45 steers randomly selected from a herd naturally infected by BPIV-3 are presented in Table I. Twelve of the screened animals which proved to be HI seronegative (titre ≤ 8) were then vaccinated with a formalin-inactivated vaccine. Serum samples taken 15 days later were examined in all the above tests (Table I, values in parentheses).

All the 57 serum samples tested for HLI-2 antibody proved to be negative (data not shown). Similar results were obtained by the HFI-2 test when sera from naturally infected steers were assayed. In postvaccination samples the reading of antibody titres was complicated due to formation of large aggregates of erythrocytes in low serum dilutions.

Table II

Comparison of HI, VN, HLI-1 and HFI-1 tests for their ability to detect any BPIV-3 antibody titre*

	HLI-1		HFI-1		VN		
	+	—	+	—	+	—	
HI +	37	8	39	6	45	0	45
—	2	10	2	10	5	7	12
HLI-1 +			34	5	39	0	39
—			7	11	11	7	18
HFI-1 +					39	2	41
—					11	5	16
					50	7	57

* results are expressed as number of sera positive (+) or negative (—) in the given test

Table III

Relationships between HI, VN, HLI-1 and HFI-1 antibody titres

Serological tests			Correlation coefficient	Regression equation
y	versus	x		
VN	vs.	HLI-1	0.807	$0.9837x + 0.1680$
VN	vs.	HFI-1	0.696	$0.8556x + 0.3207$
VN	vs.	HI	0.861	$0.8105x - 0.0702$
HI	vs.	HLI-1	0.898	$1.1631x + 0.3426$
HI	vs.	HFI-1	0.882	$1.1520x + 0.3846$
HLI-1	vs.	HFI-1	0.820	$0.8276x + 0.2012$

On the basis of titres which were arbitrarily accepted in this study as positive, the HI, VN, HLI-1 and HFI-1 tests were compared, in pairs, for ability to detect any BPIV-3 antibody titre (Table II). Fifty, 45, 41 and 39 out of the 57 sera were positive in the VN, HI, HFI-1 and HLI-1 test, respectively. Thirty-nine serum samples (68%) showed complete agreement (5 negative and 34 positive) in these assays.

The relationship between HI, VN, HLI-1 and HFI-1 antibody titres studied by linear regression analysis is presented in Table III. All correlation coefficient values were significant at the $P < 0.001$ level.

Discussion

The HI, VN, HLI-1 and HLI-2 tests as well as our HFI-1 and HFI-2 tests have been compared for their ability to detect BPIV-3 antibody in sera of naturally infected and vaccinated steers. First of all it was necessary to estimate the threshold titres to be accepted as positive. In this respect, data of the literature are limited to the HI test: usually titres of 16 or higher are considered positive (Rentschler, 1970; Morein et al., 1973). In relation to the other serological tests, in particular to our new HFI assay, threshold values had to be chosen. We found that the HI titres, in sera of both naturally infected and vaccinated animals, averaged higher than the HLI-1 and HFI-1 ones. The last two tests demonstrated approximately identical antibody levels. The antibody titres obtained by these assays in vaccinated steers were at least 4-fold higher than the corresponding prevaccination values. Linear regression analysis revealed positive correlations between HI, VN, HLI-1 and HFI-1 antibody titres. This confirms the specificity of the HFI-1 assay but suggests little diversity of the antibody titres obtained by the above techniques. Despite the high correlation, these tests were found to differ in sensitivity: the difference was particularly apparent in sera of naturally infected animals with low antibody levels. According to the titres accepted as positive, the HFI-1 and HLI-1 tests proved to be less sensitive than the routine VN and HI ones. It is necessary to consider the possible presence of non-specific inhibitors and their influence on the obtained HFI-1 results. In relation to the HI test Ketler et al. (1961) and Dawson (1963) found that nonspecific inhibitors of BPIV-3 haemagglutinin were either infrequent in, or absent from, bovine sera. In the light of data provided by Ketler et al. (1961), that inhibitors detectable by two or more serological techniques are unlikely to be non-specific inhibitors, and on the basis of the high correlation between the serological assays used in this work, the HFI-1 test can be considered specific. Nevertheless, one cannot exclude that some low HFI-1 and HLI-1 titres found in sera negative in the VN and HI tests resulted from nonspecific inhibitors of the viral F glycoprotein. This

problem needs further investigations. Although in this study no advantages of the proposed HFI-1 test over the routine HI and VN assays were observed, the HFI-1 test may find application in the case of possible vaccination with subunit vaccines containing the F glycoprotein alone.

In our attempts to detect anti-F and other non-HI antibodies, in the HLI-2 and HFI-2 tests the procedure was changed as compared to the HLI-1 and HFI-1 methods. The serum was added to the virus already adsorbed at low temperature to erythrocytes. Since at low temperature viral particles do not penetrate the cell, the inhibition by specific antibodies of the viral activities associated with its F glycoprotein was reported to be possible (Howe and Morgan, 1969; Merz et al., 1981).

Unfortunately, both the HLI-2 and the HFI-2 tests failed to detect any BPIV-3 antibody. This problem, in the light of the above data as well as our recent study with Newcastle disease virus, where we succeeded in determining the HFI-2 antibody titre in chicken sera (Trybała, 1988), remains to be clarified.

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