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BOVINE LEUKOCYTE ADHESION DEFICIENCY: A BRIEF OVERVIEW OF A MODERN DISEASE AND ITS IMPLICATIONS

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Bovine Leukocyte Adhesion Deficiency (BLAD) is a genetic disease of cattle affecting the hematopoietic system. In the last decade BLAD has become a disease of economic importance in the dairy industry. As such, this overview describes the chronological developments and thinking that led to the elucidation of BLAD as a distinct disease entity from previous models in canine and human populations. All species affected exhibit symptoms of chronic and recurrent infections. Necrotic and/or gangrenous infections of soft tissues are prevalent, as well as secondary infections with bacteria or fungi. Low birthweight and unthriftiness are key symptoms of neonates in all species affected by LAD. Dermatomycoses and impaired pus formation are also common findings. The physiological basis for BLAD is a deficiency in leukocyte (particularly neutrophil) chemotactic and phagocytic properties. The inhibition of diapedesis in the inflammatory response prevents normal immune reactions to invading pathogens. Chronic infections are a consequence of the faulty immune mechanisms. The biochemical etiology of BLAD involves cell surface glycoprotein molecules known as integrins. These are responsible for cell–cell interactions necessary for neutrophils to adhere to vascular endothelium in a normal individual. Experiments using monoclonal antibodies to block LFA-1, Mac-1, and p150,95 (three integrins vital for cell–cell interactions) mimic BLAD symptomatology and have led to the discovery of the reciprocal Intercellular Adhesion Molecule (ICAM). Through pedigree analysis and biochemical detection with restrictive endonucleases BLAD has been isolated genetically to a single gene locus. The economic significance and prophylaxis are briefly discussed. In addition, the beneficial aspects of the study of BLAD are addressed. There are advantages of producing a BLAD-like state in preventing transplant rejection, ischemia-reperfusion injury, and other scenarios arising from the deleterious effects of the inflammatory response.

Key words: Bovine Leukocyte Adhesion Deficiency (BLAD), hematopoietic system, leukocyte, neutrophil, integrins, secondary infections

Bovine Leukocyte Adhesion Deficiency (BLAD) is a relatively recently described hematopoietic disease of cattle. BLAD represents just one manifestation of a larger group of leukocyte adhesion deficiencies. LAD is broadly defined as "a

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genetic disorder of adhesion dependent leukocyte functions characterized clinically by recurrent soft tissue infections, delayed wound healing, and severely impaired pus formation despite striking blood neutrophilia" (Williams, 1990). It was first described in human patients some twenty years ago that were immunodeficient and subject to infections and neutrophil deficiencies in chemotaxis and phagocytosis. Around 1984 the etiology was determined to be a deficiency in expression of cell surface glycoproteins known as integrins (Kishimoto et al., 1987). It is these integrins which will be the primary focus of this paper. Further investigations would reveal similar syndromes in the Irish Setter and the Holstein-Friesian cow. These were designated canine and bovine granulocytopathy syndrome, respectively.

Leukocytes may be subdivided into granulocytes or agranulocytes. The former synthesize proteins which are stored in cytoplasmic granules. When stimulated, these cells can move from the bloodstream to tissues, a process called diapedesis, and release granular contents via exocytosis. Of clinical relevance to BLAD, particularly, is the neutrophil. These cells are phagocytic and contain granules with microbicidal proteins and digestive enzymes. They are involved in cell mediated cytotoxicity and antigen presentation. They have membrane receptors to recognize and bind the pathogens for phagocytosis (Williams, 1990).

Indeed, the significance of circulating polymorphonuclear cells (PMNs) in host defense mechanisms was recognized as early as the 1880's by Metchnikoff. However, it is the role of the PMNs' adherence to vascular endothelium that has been elucidated only recently (Arnaout, 1990). From the discovery of LAD in 1974 it was nearly a decade until the biochemical basis of the lack of leukocyte adhesion and migration had been ascertained.

The clinical signs of LAD in humans are recurrent necrotic or even gangrenous infections of soft tissue (subcutaneous tissues or mucous membranes). The patient with LAD is immunosuppressed and, therefore, is subject to secondary bacterial and fungal pathogens such as *Staphylococcus aureus*, *Pseudomonas*, other Gram negative enteric rods, as well as *Candida* species (Williams, 1990). There is delayed umbilical cord severance and impaired wound healing. A key feature is the presence of recurring infections despite a persistent neutrophilia. The neutrophils cannot escape from the vasculature to migrate where they are most needed, at the tissue sites of infection.

A similar condition was described in the Irish Setter in 1979 by Renshaw and Davis. A male Irish Setter with recurrent bacterial infections, associated pyrexia and persistent neutrophilia was studied. Normal values for canine leukocytes are 8,000 to 14,000/μl with 55%–75% neutrophils. Affected animals have 25,000–65,000/μl with 75%–90% neutrophils. With exacerbations of infection they can rise to 120–540,000/μl. There is a moderate normocytic, normochromic anemia. Neutrophilia can also occur in cases of granulocytic leukemia, malignant lymphoma, chemical and/or metabolite intoxication and, most commonly, from in-

fectious agents (Renshaw and Davis, 1979). In another study of an Irish Setter litter in 1979 similar clinical signs were seen. The neutrophils appeared normal ultrastructurally, lysosomes were non-remarkable, suggesting that the defect was something more subtle. Impaired *in vitro* killing of *E. coli* by affected leukocytes was observed (Renshaw and Davis, 1979). There was reduction in overall growth of affected littermates; at one month of age they were half the size of their unaffected siblings. Broad-spectrum antibiotics were necessary to keep them alive more than a few weeks to complete the study.

Other signs in the Irish Setter include omphalophlebitis, suppurative skin lesions, gingivitis, periodontitis, lymphadenopathy, pododermatitis, pneumonia, superficial pyoderma, and in advanced cases, osteomyelitis. Low body weight, dull haircoat, and multiple bone involvement was seen in another study of 12 Irish Setter pups (Trowald-Wigh et al., 1992).

In the early 1980's a LAD-like disease began to pop up in Holstein-Friesian cattle, most often seen in those between 2 weeks and 15 months (Müller et al., 1984). Hagemoser et al. (1983) describe the condition in a yearling heifer that was thin, 60% of expected size, with poor appetite and swollen denuded plaques on the muzzle. Biopsy of the nasal lesions revealed a granulomatous inflammation. In an attempt to isolate cells from the bone marrow there was iatrogenic injury to the thorax which swelled with a subsequent delay in healing (Hagemoser et al., 1983). Submandibular lymph nodes were swollen 3–5 times normal size. There was generalized lymphoid hyperplasia. In addition, splenomegaly was observed with infiltration of the red pulp with neutrophils and plasma cells. Hepatocytes had intracytoplasmic inclusion bodies which were determined to be megamitochondria. The portal triads were mildly infiltrated with neutrophils as well as them aggregating in sinusoids. Circulating leukocytes appear normal with no lysosomal inclusions. This would be a differential diagnosis for Chediak-Higashi syndrome. There was a leukocytosis, sometimes with a left shift, shortly after birth reaching levels > 100×10^9 leukocytes/L. The myeloid/erythroid ratio was 9:1. This agrees with a marked neutrophilia without concomitant anemia. In another study of 7 Holsteins focal necrotic ulcers of the nose, tongue and mucous membranes were present in all 7. All of the affected calves also exhibited lymph node enlargement, splenomegaly, thickened maxilla and mandibles, pneumonia, fibrotic pleurisy, cachexia, and lymphoid hyperplasia. In 6 out of 7 there was rumen or small intestine involvement. Bacterial pathogens (*C. pyogenes* and *P. hemolyticum*) were isolated in 2 cases from lung lesions (Takahashi et al., 1987).

Unthriftiness is a consistent finding in this disease. In one study of 8 calves there was extreme stunting of growth (40% normal body weight) and anestrus. Variability was seen, however, and some were of relatively normal stature and had normal estrus cycles (Müller et al., 1984). Those with stunted growth often have large heads and hooves for their size (Jorgensen et al., 1993*b*). Dermatomycoses,

particularly ventral dermatitis and vasculitis, are often seen. Lack of pus formation is also a prominent feature. In a study of 14 cases between 1977 and 1991 by Gilbert et al. (1993), in addition to those signs normally reported, they noted bruxism, and wounds associated with dehorning or ear tagging that were still suppurative 6 months after the procedure! A survey of many studies reveals mainly gastrointestinal and respiratory involvement in the infectious pathogenesis of this condition. The molecular basis of bovine granulocytopathy syndrome (BGS) was found to be, as in human LAD or canine LAD, a deficiency in integrins. These have been studied extensively in recent years. BGS has since been renamed BLAD (Kehrli et al., 1990).

The biochemical basis of all LAD syndromes is an inherited (autosomal recessive) deficiency in cell surface glycoproteins known as integrins. The integrins are one of three families of cell surface molecules regulating migration of leukocytes and mediating the immune response. The Immunoglobulin superfamily includes receptors for T and B cells. Selectins, the third group, are necessary for lymphocyte and neutrophil interaction with vascular endothelium (Springer, 1990). The integrins are cellular adhesion/recognition mechanisms not only important in diapedesis and inflammation but also in embryological differentiation and development (Kishimoto et al., 1992). Evolutionarily these molecules are related to other integrins such as fibronectin receptors and platelet gp IIb/IIIa. These guide cells during embryogenesis and normal healing processes. Integrins of importance to BLAD are LFA-1, Mac-1, and p150,95. Leukocyte function associated antigen (LFA-1), macrophage antigen (Mac-1) and p150,95 are heterodimers each consisting of a unique α subunit (CD11a, CD11b, and CD11c, respectively). All 3 share a common β subunit (CD18) (Springer, 1990). Hence, there is a redundancy here; all 3 contribute to neutrophil and monocyte adhesion to endothelial cells and substrates. No selective deficiency of just one integrin is observed (Kishimoto et al., 1992). The α and β subunits are noncovalently associated. They are transmembrane proteins with relatively small cytoplasmic domains. There is evidence that they are intrinsically involved in connections with the cytoskeleton (Haynes, 1987). It seems that the β unit distributes with extracellular fibronectin and intracellular components of the cytoskeleton (vinculin, talin, actin, α actinin, and tropomyosin). The CD11a, CD11b, and CD11c molecules are 180, 155, and 150kd proteins while the β subunit is 94kd. Divalent cations (Ca^{2+} + Mg^{2+}) are essential for maintaining the structural integrity and function of these $\alpha\beta$ complexes (Arnaout, 1990). The CD11a/CD18 is expressed on all leukocytes regardless of subtype. CD11b/CD18 and CD11c/CD18 are found on the cell membranes of monocytes, macrophages, PMN's, and NK (natural killer) cells. In addition, CD11c is found on some CD5 B cell lines and some activated lymphocytes. It is a marker for hairy cell leukemia (Kishimoto et al., 1992). Normally T and B cells

express CD11a/CD18. Active granulocytes express CD11b/CD18 in greater proportion than the other cell adhesion molecules. A resting macrophage, for example, expresses CD11a most abundantly, followed by CD11b and then CD11c. An activated tissue macrophage expresses CD11c > CD11a > CD11b (Arnaout, 1990). There are synonymous designations for both Mac-1 and p150,95. Mac-1 is also known as Mo-1, OKM-1, or complement receptor type 3 (CR3). p150,95 can also be referred as CR4 or LeuM5 (Kishimoto et al., 1987).

Experimental blocking of LFA-1, Mac-1, and p150,95 with monoclonal antibodies (Mabs) reveals the physiological consequence of deficiencies in these molecules. Mab to LFA-1 blocks leukocyte adhesion to endothelial cells, inhibits conjugation formation required for antigen presentation, cytotoxic T cell killing, T cell proliferation, and NK cell activity. Mabs to Mac-1 block binding to iC3b (fragment from cleavage of C3b, third component of complement) coated particles, myeloid cell adhesion to endothelium, and neutrophil homotypic aggregation and chemotaxis. Mabs to p150,95 block monocyte adhesion to endothelial cells and cytotoxic T cell conjugate formation with target cells. These Mab studies led to the discovery of ICAMs (intercellular adhesion molecules) as counter receptors to the integrins (Diamond et al., 1990). ICAMs are also transmembrane proteins but of the immunoglobulin superfamily. They have 5 immunoglobulin-like domains in the extracellular portion of the molecule. ICAM-1, LB2 (CD54) and ICAM-2 bind to CD11a/CD18. ICAM-1 and ICAM-2 are counter receptors for LFA-1. ICAM-2 is expressed constitutively on endothelial cells. ICAM-1 will bind to Mac-1. These binding interactions facilitate adhesion between the neutrophil and the endothelial cell (Springer, 1990).

The exact mechanisms that allow diapedesis to occur in normal leukocytes during inflammatory events are complex and poorly understood. However, studies of BLAD have opened up new ideas and speculation in these matters. There seem to be two classes of adhesion receptor interactions differentiated by the distance between plasma membranes of the 2 closely opposed cells. T cell receptor (TCR) and MHC (major histocompatibility complex) and CD2-LFA3 are close (about 13 nm or less) whereas LFA-1 with ICAM-2 or ICAM-1 are more distant (27 and 36 nm, respectively). The "close" type requires extensive interdigitation of membrane glycocalyces of the 2 adhering cells and is thought to hinder lateral mobility of surface glycoproteins and would exclude major glycocalyx components CD43, CD45 from this zone. Conformational changes could affect cell signalling. The "far" type has less glycocalyx interdigitation compatible with lateral movement of one membrane surface over another without the frictional drag of the interlocking glycocalyces. In essence, there is less steric hinderance. Cells can have focal contacts 10–15 nm apart during adherence. During migration close contacts (30 nm) are established with the substrate. Lipids, membrane proteins and other cellular components would be drawn forward in extensions of the leading edge of the cell. Integrins may serve as transiently fixed points in the membrane connecting the

cytoskeleton to extracellular environment. The hypothesis is that there are localized changes in membrane avidity which drive migration by alternative tension and relaxation of cytosketal elements (Springer, 1990).

Human LAD, canine LAD, and BLAD are all the result of a mutation in the β subunit (CD18). The heterogeneity of the human condition is associated with varieties of mutations within the same gene locus. These defects have been isolated to chromosome 21. The α subunits of LFA-1, Mac-1, and p150,95 are within bands p11–p13.1 of chromosome 16. Kishimoto et al. (1987) recognized 5 phenotypes using Southern blot analysis. Some patients had undetectable β subunit MRNA and protein precursors. The gene was present, thus, there was a mutation. Others had low levels, synthesizing some 10% of the normal amount of α and β complexes. In addition, one sees aberrantly large or small β subunit precursors. The last group had normal sized proteins. Defects affect either β subunit synthesis or the ability for α and β subunits to associate with each other. (Impaired glycosylation affects the tertiary structure of the β subunit.) This conformational change disallows interaction between the two subunits and there is a resultant loss of expression on the cell membrane. In severe deficiencies there is < 1% expression; moderate deficiencies are those in which 5–10% normal amount of integrins are present. Children with severe phenotypes often die as infants < 2 years old due to immunosuppression and secondary bacterial infections. Moderate phenotypes can survive to adulthood (Kishimoto et al., 1987).

In 1970, Renshaw revealed autosomal recessive inheritance in the Irish Setter by pedigree analysis. Backrossing affected individual with his dam creates litters with both affected and normals (Renshaw and Davis, 1979). A normal unrelated female mated with the propositus (the individual in the study) results in normal offspring. Subsequent breedings, however, produced affected males and females. Observed ratios were close to expected 1:3 when heterozygotes mate. Canine granulocytopathy syndrome was the first LAD with no lysosomal aberrations observed in a species other than humans (Giger et al., 1987). The discovery of the significance of LEU-M5 (p150,94) to CGS was by Giger et al. (1987). They described a mating between an 18-month-old Irish Setter female cross breed. She was 1 of 5 offspring of an accidental breeding between a mother and son. This indicates the importance of educating breeders about this disease to avoid matings of carriers.

Point mutations of the gene coding for CD18 are implicated in the etiology of BLAD. DNA studies done with a restriction endonuclease (*Taq* I) reveal differences between healthy and affected calves. Normal animals have fragments of 100, 200 and 300 base pairs whereas the BLAD calves have 200 and 400 bp bands. The donor was healthy and had all fragment lengths, and was, therefore, a carrier for the defect. The carrier had both normal and abnormal chromosomes. The sire was identical in this regard to the dam. Using polymerase chain reaction

(PCR) techniques one can detect carriers which are heterozygous for the abnormal CD18 gene (Kehrli et al., 1990). The deficiency seen in the Holstein-Friesian cattle is a deficiency in Mac-1. There is a single point mutation, a substitution of guanine for adenine at position 383 in the cDNA of the CD18 gene (Shuster et al., 1992). This results in a substitution of guanine for aspartate at amino acid #128 in the protein. There is another mutation; a substitution of a cytosine for thymine. However, this mutation is silent. A bull named NJY Hubert sired 17% of registered calves born in 1991 and was involved in 820 of 1183 carrier and carrier matings observed. He was mainly responsible for the spread of BLAD in Denmark (Jorgensen et al., 1993*a*). Imported semen of American Holstein bulls had an enormous impact on the Netherlands in the past decade. Sires of both affected animals in another study were linked to an ancestral sire carrier of bovine D128G CD18 allele. In yet another study of 20 calves the incidence was found to be 15% in bulls and 6% in cows, overall incidence of 0.2% of Holsteins at birth, in the U.S. (Shuster et al., 1992). BLAD is a common disease throughout the world, one of the most significant diseases in animal agriculture today. In Japan and Europe many cases can be traced back to "Osborndale Ivanhoe", designated the Father of the Holstein breed due to his prolific nature.

BLAD calves usually die at < 1 year. Survivors will exhibit poor growth and low reproductive ability. In the U.S. 80% of 10,000,000 dairy cows are Holsteins. About 16,000 calves with LAD are born per year. Average economic loss is $300/calf. This means that there is a $5,000,000 loss of BLAD in the U.S. alone per year.

Treatment considerations consist of supportive treatment and prophylaxis with antibiotics (trimethoprim-sulfa) for bacterial infections. Mild phenotypes may be maintained for a while using this method. However, the disease is usually rapidly fatal. The only curative treatment is somatic cell reconstitution and bone marrow transplants (Arnaout, 1990). LFA-1 function has been restored in lymphoblastic cell lines from human LAD patients using CD18 cDNA in an Epstein-Barr viral vector or a retroviral vector (Kehrli et al., 1990).

The study of BLAD offers many implications for further investigation and beneficial applications. The carrier state is helpful to isolate genetic traits (other than LAD) on CD18. Selection against Weaver Syndrome, a heritable neurologic disease in Brown Swiss Cattle is now possible. Fascinatingly, there seem to be beneficial effects on ischemia-reperfusion injury. Acquired deficiencies can be induced by administering Mabs to CD11/CD18. The antibodies will attenuate tissue destruction associated with myocardial infarctions in dogs, intestinal ischemia (cats), and hemorrhagic shock syndrome. It reduces tissue injury, microvascular permeability, and acidosis. Another positive application is in the use of Mabs to integrins to prevent transplant rejection reactions (Arnaout, 1990).

Since today's dairy industry breeds for lactational performance, there is a lot of genetic relatedness in the Holstein. Only a few sires fertilize many cows, however, this makes the eradication of the disease easier. Current screening programs with elimination of affected sires from the breeding pool should drastically reduce the incidence of BLAD (Shuster et al., 1992). With strict adherence to these protocols we can be confident that the prevalence of BLAD will diminish and the syndrome will become a relic of the past.

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ENTEROTOXIGENIC *ESCHERICHIA COLI*, ROTAVIRUS, PORCINE EPIDEMIC DIARRHOEA VIRUS, ADENOVIRUS AND CALICI-LIKE VIRUS IN PORCINE POSTWEANING DIARRHOEA IN HUNGARY

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In order to obtain data about the significance of enterotoxigenic *E. coli* (ETEC), and of the different enteric viruses in the aetiology of porcine postweaning diarrhoea, bacteriological, electron microscopic and ELISA studies were made on dead and live pigs. *E. coli* from the small intestine of diarrhoeal weaned pigs that died were tested for serogroups, pili (fimbriae) and toxin-genotype. The 108 haemolytic *E. coli* representing 14 farms and 42 pigs were typed as follows: 0149:K88$^+$ETEC (56.5%), OX:K88$^+$ETEC (21.3%), O141:F18ac$^+$ ETEC (4.6%), O147:F18ac$^+$ETEC (3.7%), OX and O157:F18ac$^+$ETEC (9.3%), verotoxigenic O141:F18ac (2%). In another study, when faecal samples of 92 live diarrhoeal weaned pigs (representing 19 farms) were tested, rotaviruses (18.6%), porcine epidemic diarrhoea virus (PED) (5.5%) and calici-like viruses (5.5%), and adenovirus (two pigs) were detected, besides K88$^+$ETEC (12%) and K99$^+$ or 987P$^+$ETEC (one of each). Combined infections were detected in 9% of the samples. Sequential studies of diarrhoeal and nondiarrhoeal weaned and unweaned pigs indicated that PED virus and group A rotavirus were related to diarrhoea but adeno- or calici-like virus were not. It was concluded that K88$^+$ETEC was the overwhelming aetiologic agent of porcine postweaning diarrhoea in Hungary, but F18ac$^+$ETEC, group A rotavirus and PED virus were also significant.

Key words: Enterotoxigenic *E. coli*, enteric viruses, rotavirus, PED-coronavirus, adenovirus, calicivirus, pigs

Significant aetiologic agents of porcine diarrhoea include enterotoxigenic *Escherichia coli* (ETEC) (Fairbrother, 1992), rotavirus (Paul and Stevenson, 1992), transmissible gastroenteritis (TGE) (Saif and Wesley, 1992) and porcine epidemic diarrhoea (PED) virus (Pensaert, 1992) in both suckling and weaned pigs. Besides, sporadic reports exist on the occurrence of calici-like viruses (Shirai et al., 1985) and adenovirus in newborn pigs (Derbyshire, 1992), but not in weaned pigs.

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In Hungary there have been several reports about the significance of K88$^+$ETEC in weaned pigs (Pesti and Semjén, 1974; Semjén and Szabó, 1976). Recently a new fimbrial antigen, F18ac (Rippinger et al., 1995), previously designated 2134P (Nagy et al., 1992), has also been reported on several Hungarian postweaning ETEC isolates (Dean-Nystrom et al., 1993; Nagy et al., 1996), but their distribution within herds has not been investigated so far.

The age-related emergence of rotavirus antibodies in pig herds has been reported by Mocsári et al. (1982) in Hungary, but data about rotavirus shedding and about the type and significance of the virus present have not been available. Similarly, detection of, and experimental infection with PED virus was reported by Horváth and Mocsári (1981) in suckling pigs but studies on the prevalence and significance of the virus in weaned pigs in Hungary are missing. Adenovirus enteritis was diagnosed in Hungary in diarrhoeal newborn pigs (Mocsári, 1984, personal communication) but so far no reports have been available about adenovirus in weaned pigs.

In order to obtain relevant data for further use in prevention of the disease, these studies aimed to gain data about the prevalence and significance of different adhesin and toxin types of ETEC and about the involvement of the enteric viruses in porcine postweaning diarrhoea in Hungary.

Materials and methods

E. coli bacteria

One set of 108 *E. coli* was isolated from the small intestines of 42 weaned pigs that died as a result of diarrhoea. These pigs represented 14 farms with at least two pigs per farm. In another study (involving 92 pigs from 19 herds) faecal samples of live diarrhoeal weaned pigs were investigated. Typing included investigation of serogroups for the most common porcine types and determination of pilus (fimbrial) antigens K88, K99, 987P typical of pigs (Ørskov and Ørskov, 1978), as well as for the recently described fimbrial antigen variants F18ab and F18ac (Rippinger et al., 1995), using absorbed antisera and — in the case of F18ac — monoclonal antibodies (Dean-Nystrom et al., 1993). Enterotoxin types were tested using colony blot DNA hybridization as described (Nagy et al., 1990). Verotoxicity was determined in Vero cell cultures (Konowalchuk et al., 1977) and by colony blot DNA hybridization using the Shiga-like toxin (SLTI and SLTII) DNA probes (Newland and Neill, 1988). In a second investigation of ETEC, faecal samples of 92 live diarrhoeal weaned pigs were tested and selected strains of haemolytic *E. coli* were typed.

Viruses

The study on viruses involved faecal samples of 92 live diarrhoeal weaned pigs from 19 farms. In this study, detection of rota-, TGE, PED, adeno- and calici-like viruses was done by transmission electron microscopy (Almeida et al., 1978). Confirmation of the electron microscopic findings was done by immunofluorescence using FITC labelled conjugates (specific to TGE, rota- and PED viruses), by latex agglutination (Wellcome) and ELISA (in the case of rotavirus), and by testing seroconversion (by ELISA for rotavirus and by counter-immunoelectrophoresis for adenovirus).

Serogroup of rotavirus was determined by immune electron microscopy using group A rotavirus antiserum raised in SPF calves (kindly provided by Dr. V. Pálfi). The specimens were reacted (overnight, 4 °C) with 1:20 dilution of the serum, washed 3 times with dH_2O, stained with 2% phosphotungstic acid (pH 7.0), and viewed at 80 kV with an electron microscope. Samples treated with fetal calf serum served as negative controls.

In one herd, sequential studies were performed on rotavirus shedding using ELISA as follows. Plates sensitized with 1:4000 dilution of the above group A rotavirus antiserum were covered with 1:5–1:10 dilution of faecal supernatants, incubated (1 h, 22 °C) and, after having been washed 3 times, reacted (30 min, 22 °C) with anti-rotavirus conjugate prepared for these studies from the above antiserum as described (Nakane and Kawaoi, 1974). Plates were washed and substrate (OPD) added (10 min, dark) and then stopped (4N H_2SO_4). Calf rotavirus served as positive control. The reaction was regarded as positive if the absorbance reached $\geq 2\times$ of that obtained for the negative control. ELISA positive samples were run parallel with electron microscopy in approx. 50% of the cases. At two different weanings 10 pigs were sampled weekly on each occasion. In experiment II the same pigs at the same times were also tested for rotavirus antibodies using a blocking version of the above ELISA system with bovine rotavirus as test antigen (prepared as described by Mocsári et al., 1982). Reciprocal values of the highest dilution of sera that reached a positive level were regarded as titres. Serum dilution was regarded as positive at extinction values below 1.00.

Serological investigation of 10 pigs from the group which had shed adenovirus 3 weeks earlier was performed by counter-immunoelectrophoresis (CIEF) as described (Mocsári et al., 1982) using a bovine adenovirus type 2 isolate as mammalian group antigen (kindly provided by Dr. V. Pálfi).

In another herd, the shedding of PED virus and calici-like virus before and after weaning (between 5–6 weeks of age) was studied, by sampling the same 8 pigs from two litters at one-week intervals.

Results

The first series of studies — involving 108 *E. coli* strains from 42 pigs of 14 herds — resulted in the detection of O149:K88$^+$ETEC (56.5%), OX:K88$^+$ETEC (21.3%), O141:F18ac$^+$ETEC (4.6%), O147:F18ac$^+$ETEC (3.7%), OX and O157:F18ac$^+$ETEC (9.3%), Shiga-like toxigenic *E. coli* of O141:F18ac$^+$ETEC (1.8 %) and nonenterotoxigenic *E. coli* (2.8%) as summarized in Table 1. The main finding of this survey was, however, that *E. coli* isolates from half of the herds were quite homogeneous (almost only O149:K88$^+$ETEC) while the other half of the herds had both K88$^+$ETEC and F18ac$^+$ETEC (except one herd where 4 strains from 2 pigs were all O147:F18ac$^+$ETEC). Two strains (O141:F18ac$^+$) produced Vero cytotoxin and hybridized with the SLTII probe.

When faecal samples of 92 live diarrhoeal pigs from 19 farms were investigated, ETEC was much less frequently detected than above (K88$^+$ETEC in 12% and K99$^+$ETEC and 987P$^+$ETEC in one pig each). Regarding viruses of these samples, rotavirus (18.6% from 8 herds), adenovirus (in two pigs of one herd) calici-like virus particles (5.5% from one herd), and PED virus (5.5% from two herds) were detected. TGE virus was not detected in any of the cases.

Adenovirus was diagnosed in a herd where 2 days after weaning 2 of 4 diarrhoeal pigs shed typical adenovirus particles of hexagonal shape (Fig. 1). Sera of 10 pigs from this group, when tested by CIEF, were found to contain antibodies to the Mastadenovirus genus in all samples.

Calici-like virus particles were approx. 30 nm in diameter and were characterized by typical hollows and cup-shaped depressions (Fig. 2).

Combined infections (in 9% of the faecal samples encountered in this survey) were detected as follows: O147 ETEC + rotavirus (2); K88$^+$ETEC + rotavirus (4); O149:K88$^+$ETEC + rotavirus + adenovirus (1); O149ETEC + rotavirus + PED virus + calici-like virus (1).

Rotavirus from a faecal sample of a pig in one herd was investigated by immune electron microscopy using group A rotavirus antiserum and was found to produce specific immuno-aggregates (Fig. 3A and Fig. 3B). Rotavirus shedding and humoral rotavirus antibodies of pigs of that herd were studied in two subsequent weanings by weekly ELISA investigation of 10 pigs in each. The results are summarized in Table 2. It should be noted that in experiment IIA none of the tested pigs had diarrhoea for two weeks after weaning, while at the same time in experiment IIB, 3 out of 4 rotavirus-positive pigs had diarrhoea, and only one of these had both haemolytic *E. coli* (O157) and rotavirus, while the other two shed rotavirus only. Persistent rotavirus shedding occurred in two cases: in one clinically healthy pig in both groups IIA and IIB (Table 2). The presence of rotavirus additional to ETEC in this herd was accompanied by high losses (11–25 %) due to

postweaning diarrhoea. As a result of introducing barley diet between baby feed and weaner feed (as described in experiments I and IIA), the losses due to postweaning diarrhoea dropped to 1–2% in that herd.

Table 1

Dominant sero- and toxin types of *Escherichia coli* on 14 farms based on the examination of pigs that died of postweaning diarrhoea

Farm	No. of pigs tested	*E. coli* isolate tested		
		No.	Serotype*	Toxin**
1	2	4	O149:K88ac	LT, STb
		1	OX:K88	NT
2	2	6	O149:K88	NT
3	2	6	O149:K88	NT
4	2	6	O149:K88	NT
5	2	6	O149:K88	NT
6	2	6	ONT:K88	NT
7	3	7	ONT:K88	NT
8	5	7	O149:K88	NT
		2	O141:F18ac	STa,b
		2	O8	Tox^-
9	4	8	O149:K88	NT
		1	O141:F18ac	STa,b
		1	O157:F18ac	STa,b
		2	OX:F18ac	NT
10	4	8	O149:K88	NT
		3	OX:K88	NT
		1	O162	Tox^-
11	7	7	O149:K88	NT
		6	OX:K88	NT
		4	OX:F18ac	STa,b
12	3	2	O149:K88ac	LT,STa,b
		2	O141:F18ac	STa,b
		2	O141:F18ac	SLTIIv
13	2	1	O149:K88	NT
		3	OX:F18ac	STa,b
14	2	4	O147:F18ac	STa,b

*: K88ac variants determined on selected strains; **: toxin type determined by DNA hybridization; Tox^-: nontoxigenic; NT: not tested; SLTIIv: Shiga-like toxin type II variant (verotoxin); O antigen not tested (ONT), or could not be determined (OX)

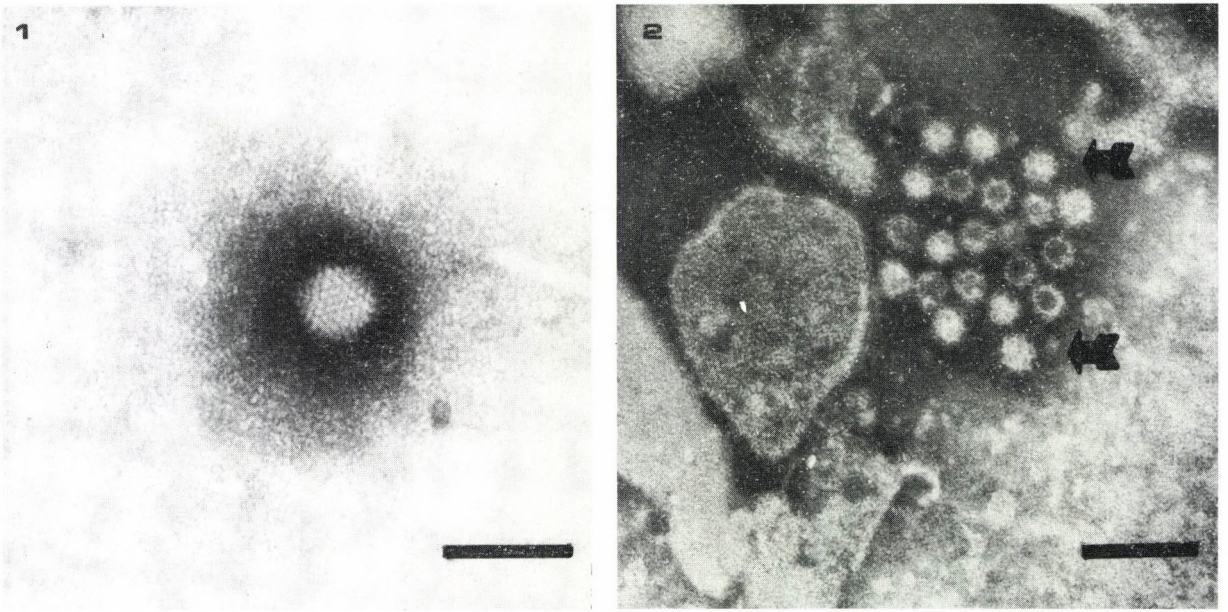


Fig. 1. Transmission electron micrograph of a typical adenovirus particle from the faeces of a diarrhoeal weaned pig. Bar equals 100 nm

Fig. 2. Transmission electron micrograph of a group of complete and incomplete calici-like virus particles from a diarrhoeal weaned pig. Note the typical cup-like depressions in the complete particles (arrows). Bar equals 100 nm

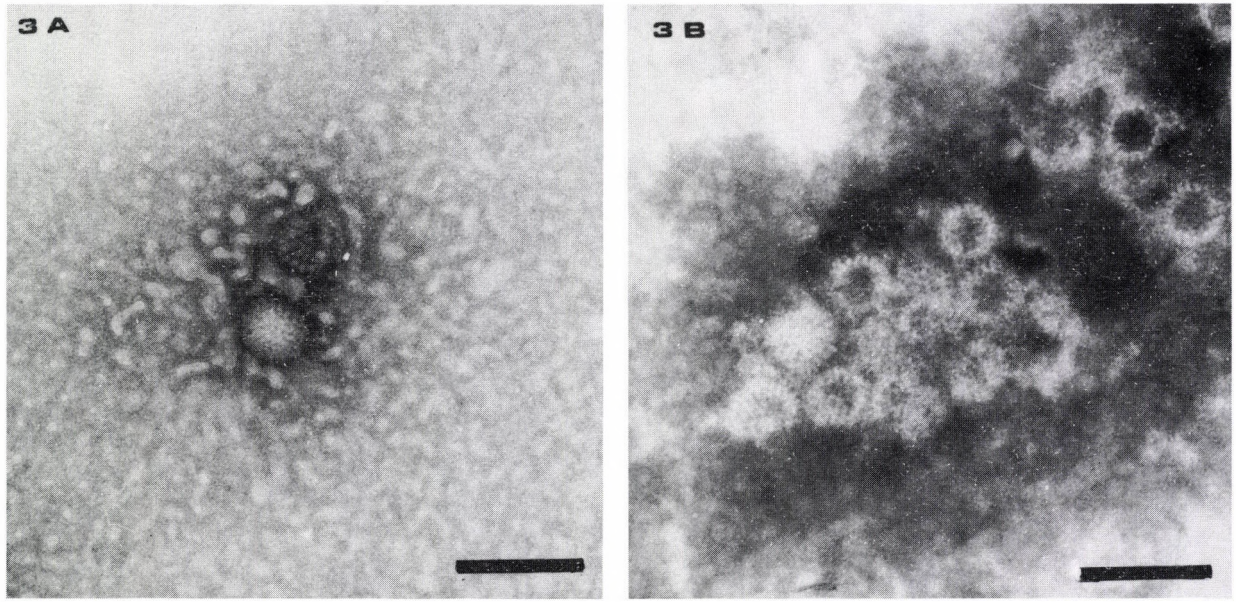


Fig. 3A. Transmission electron micrograph of two rotavirus particles (one complete, one incomplete) derived from a diarrhoeal weaned pig and treated with fetal calf serum (as described for the negative control in Materials and Methods). Bar equals 100 nm

Fig. 3B. Immune electron microscopic picture of rotavirus particles from the same sample as 3A. Virus particles are aggregated by calf-derived group A rotavirus antibodies (surrounding and partially covering the particles as an electron dense material). Bar equals 100 nm

Table 2

Results of bacteriological and ELISA studies on faecal shedding of rotavirus and haemolytic *E. coli* and mean rotavirus antibody titres of pigs in the first 3–4 weeks after weaning

	Weaning	Week 1	Week 2	Week 3	Week 4
*Exp. I**					
HEc**	0/3	5/10	1/10	0/10	NT
Rota	3/3	2/10	8/10	7/10	NT
*Exp. IIA**					
Diarr.	0/5	0/5	0/5	0/5	0/5
HEc**	0/5	1/5	0/5	0/5	0/5
Rota	0/5	0/5	1/5	1/5	1/5
*Exp. IIB**					
Diarr.	0/5	3/5	0/5	0/5	0/5
HEc**	1/5	2/5	1/5	0/5	0/5
Rota	1/5	4/5	1/5	1/5	1/5
Anti-rota titres	1:8	1:30	1:50	1:90	1:40

* In experiments I and IIA feeding involved baby feed for 5 days post weaning, which was followed by 3 days of barley feeding and by a gradually increasing ratio of regular weaner diet for 8 days thereafter.
In experiment IIB, the baby feed was followed by a regular diet from the 5th day after weaning.

** Haemolytic *E. coli* typing of selected isolates revealed OX:K88 (one strain), O138:K88 (3 strains), O157 and OX (one strain each, without known adhesin)

Shedding of calici-like and other viruses was monitored in 8 pigs (representing 2 litters in one herd, under normal farm conditions) between 2 and 9 weeks of age. Pigs were weaned at 6 weeks. Table 3 summarizes the results obtained on the shedding of calici-like, rota- and PED virus. (These data on shedding rates are not included in the prevalence rate reported above.) It seems that rotavirus and PED virus infections were the most persistent. One pig persistently shed PED coronavirus, had diarrhoea for 5 weeks from 4 to 9 weeks of age, and became runted.

In diarrhoeal pigs of this group the following diagnoses were made. Week 2: PED (3), calici (1) and O149 ETEC (1), none (1); week 3: none (2); week 4: PED + rota (2); week 5: PED + rota (1); week 6: PED (1), HEc (1); week 7: PED (2); week 8: PED (1); week 9: PED (1).

Table 3

Shedding of calici-like, rota- and porcine epidemic diarrhoea (PED) viruses as well as haemolytic *E. coli* (HEc) before and after weaning under normal farm conditions

	Age of pigs (in weeks)							
	2	3	4	5	6	7	8	9
Diarrhoea:	4/8	2/8	3/8	1/8	2/8	2/8	1/8	1/8
HEc:	2/8	0/8	0/8	0/8	4/8	0/8	0/8	0/8
Viruses:								
Calici	2/8	0/8	0/3*	0/8	0/8	2/8	0/1*	0/1*
Rota	3/8	1/8	1/3*	1/8	0/8	0/8	0/1*	0/1*
PED	6/8	1/8	1/3*	1/8	1/8	4/8	1/1*	1/1*

*Only diarrhoeal pigs were tested

It seems that PED coronavirus was the most important postweaning diarrhoeal agent in this group. Calici-like viruses were probably not playing a significant role in diarrhoea of these pigs: they were found at 2 weeks of age and one week after weaning, mostly in nondiarrhoeal pigs.

Discussion

Most of the *E. coli* strains studied here in detail represented the classical O149:K88$^+$ETEC, confirming earlier observations of Semjén and Szabó (1976). In general, K88$^+$ETEC was the dominating ETEC type in half of the farms. However, there were ETEC with the newly recognized and classified F18ac adhesins — with or without K88$^+$ETEC — in the other half of the herds with postweaning diarrhoea. These F18ac$^+$ETEC strains usually produced ST toxins for which DNA colony hybridization has proven to be a more humane and more convenient method as compared to classical phenotyping (mouse inoculation and ligated intestinal loop tests).

The observations published on the prevalence and significance of porcine rotavirus infection in Hungary have so far been limited to the serological survey conducted by Mocsári et al. (1982). Here we have confirmed these reports about the high prevalence of rotavirus infection in pigs, and extended earlier observations by calling attention to pigs that may shed the virus persistently. Additionally, our ELISA study indicated a mobilization of the group A rotavirus in weaned pigs (increased frequency of virus shedding and increased antibody titres during 3–4 weeks after weaning), suggesting a pathogenic role of group A rotavirus in porcine

postweaning diarrhoea. At present it seems that feeding and management techniques can be best used in the prevention of rotavirus-induced diarrhoea in weaned pigs (Lecce and King, 1981).

Before this study, no information was available on the occurrence of calicivirus in Hungary. The data reported here indicate a situation similar to what was described for the US (Saif et al., 1980) and Japan (Shirai et al., 1985) for rotavirus and for calici virus.

As regards porcine adenovirus, Derbyshire et al. (1966) reported the excretion of this virus in the postweaning period but there have been very few confirmations of his findings since. Our observations should be one of them. As these viruses were present in many cases in the form of mixed infections, the judgement on their significance must be made with caution. This is especially true for adenovirus and calici-like viruses, which have been known to affect primarily young suckling pigs (Saif et al., 1980; Derbyshire, 1992). Although no firm conclusion could be drawn regarding the pathogenic role of adenovirus and calici-like virus in our cases of postweaning diarrhoea, the sporadic occurrence of these viruses in weaned pigs indicates a negligible role.

In contrast, the information collected here indicates that group A rotavirus has frequently been involved in the aetiology of postweaning diarrhoea in pigs in Hungary, and the association of PED virus with pre- and postweaning diarrhoea also suggests its pathogenic role where present.

Finally, our data have led us to the conclusion that $K88^+$ETEC and $F18ac^+$ETEC are the two most important types of *E. coli* in the aetiology of porcine postweaning diarrhoea in Hungary. Group A rotavirus (and occasionally PED coronavirus) are also significant — but less investigated — viral components of this disease complex. Therefore, future vaccination programs for the prevention of porcine postweaning diarrhoea should consider K88 and F18 *E. coli* adhesins as bacterial and porcine group A rotavirus as viral components for general use.

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MYCOPLASMAS ASSOCIATED WITH BOVINE CONJUNCTIVITIS AND KERATOCONJUNCTIVITIS

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In two separate herds of fattening calves a sudden-onset outbreak of ocular disease with profuse lacrimation occurred. The disease resembled the early stage of infectious bovine keratoconjunctivitis but after a few days the clinical signs of bronchopneumonia appeared. From conjunctival swabs *Mycoplasma (M.) bovigenitalium, M. bovirhinis* and infectious bovine rhinotracheitis (IBR) virus were isolated. *Moraxella bovis* infection was not established. In one of the herds *M. bovigenitalium* was also found in the pneumonic lungs of dead calves. In one herd *M. bovoculi* was isolated from a cow with chronic keratoconjunctivitis, housed together with affected calves. Mycoplasmas were not isolated from ocular swabs of six bulls originating from a Reproductive Centre with temporary occurrence of unilateral serous conjunctivitis resistant to antibiotic therapy.

Key words: Bovine eyes, *Mycoplasma bovigenitalium, M. bovirhinis, M. bovoculi,* IBR virus

Various bacterial, viral and parasitic agents have been found in cattle in association with infectious bovine keratoconjunctivitis (IBK) or conjunctivitis (Rosenbusch and Knudtson, 1980; Punch and Slatter, 1984). According to reviews by Punch and Slatter (1984), Barber et al. (1986) and Ross (1993), *Moraxella bovis* is the most important pathogen involved in IBK. However, many other organisms isolated from affected bovine eyes cause a conjunctivitis rather than keratoconjunctivitis. Among the organisms proposed as causative agents of bovine ocular disease, various mycoplasmas and acheloplasmas are quoted too, although their aetiological significance still remains obscure because of their occurrence in healthy and clinically affected animals (Schöttker-Wegner et al., 1990). The present paper reports the isolation of *Mycoplasma bovigenitalium, M. bovirhinis, M. bovoculi* and infectious bovine rhinotracheitis (IBR) virus from cases of conjunctivitis or keratoconjunctivitis in cattle.

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Materials and methods

In two separate herds (A and B), each comprising 30 fattening calves of the Simmental breed, 5 to 6 months of age, a sudden spread of an ocular disease suggestive of the early stage of IBK was observed during the winter period. Approximately 60% of the animals got affected on both eyes with profuse lacrimation. Their conjunctivae were hyperaemic and oedematous with a copious serous discharge. The cornea was not affected. Two or three days later, the affected calves developed clear-cut clinical signs of bronchopneumonia. In herd A a 8-year-old cow with previous irreversible ocular changes was housed together with calves. This cow did not show the signs of bronchopneumonia or lacrimation, however, corneal opacity of both eyes with subsequent blindness was observed. Furthermore, six bulls originating from a Reproductive Centre with temporary occurrence of unilateral serous conjunctivitis resistant to antibiotic therapy were examined.

For microbiological examination, conjunctival swabs were taken from both eyes of three calves from each herd, including the cow from herd A and the 6 bulls from the Reproductive Centre. In addition, lung tissues of three dead calves from herd A were investigated only for mycoplasma.

All the swabs were thoroughly rinsed in 3 ml of nutrient broth and these materials were transported to the microbiological laboratory within 2 h. For isolation of bacteria and mycoplasma the materials were inoculated on nutrient agar supplemented with horse serum and PPLO broth. For virus isolation secondary bovine kidney cells were inoculated with ocular materials from each herd of calves. Conjuctival swabs from the bulls were not examined virologically. After two days of incubation at 37 °C in an atmosphere of 10% carbon dioxide in air, the inoculated agar plates were examined and the bacteria grown out were identified according to Krieg and Holt (1984) and Sneath et al. (1986). PPLO broths inoculated with different dilutions of original material were incubated at 37 °C and, after three and seven days of incubation, plated on PPLO agar (Hayflick, 1965). Biochemical characterization was done according to Razin and Tully (1983) and the isolated mycoplasma strains were identified by indirect immunofluorescence (Rosendal and Black, 1972). The cell cultures incubated at 37 °C were examined for cytopathic effect (CPE) every day. When CPE developed, the presence of IBR virus was confirmed by immunofluorescence test performed according to Shipper and Chow (1968).

Results and discussion

Moraxella bovis was not isolated from the ocular materials of any examined animal. In the conjunctival swabs of four calves a small number of α-haemolytic streptococci was found. From the swabs of bulls from the Reproductive Centre no pathogenic bacteria or mycoplasma were isolated.

IBR virus was demonstrated in fattening calves originating from both herds (Table 1). Mycoplasmas were isolated from both eyes of two calves from each herd and from the eyes of the cow. The isolated strains gave faint or negative reaction by growth inhibition tests (Clyde, 1964) and their identity was confirmed by immunofluorescence. Three strains isolated from calves were determined as *M. bovigenitalium* and one as *M. bovirhinis*. The isolate from the cow was identified as *M. bovoculi*. The biochemical and cultural characteristics of isolated mycoplasmas were consistent with the known properties of each identified species (Krieg and Holt, 1984). The identification of isolated mycoplasmas was confirmed by the Mycoplasma Reference Laboratory, Public Health Laboratory Service, London.

Table 1

Clinical and microbiological findings

Clinical signs	Animal	Material	Mycoplasma	Virus
1. Catarrhal conjunctivitis Bronchopneumonia	Herd A			
	Calf No. 1	Ocular swab	*M. bovigenitalium*	
	2	Ocular swab	*M. bovigenitalium*	
	3	Ocular swab	Negative	
	4	Lung tissue	*M. bovigenitalium*	IBR
	5	Lung tissue	*M. bovigenitalium*	
	6	Lung tissue	*M. bovigenitalium*	
	Herd B			
	Calf No. 1	Ocular swab	*M. bovigenitalium*	
	2	Ocular swab	*M. bovirhinis*	IBR
	3	Ocular swab	Negative	
2. Infectious bovine keratoconjunctivitis	Cow	Ocular swab	*M. bovoculi*	ND
3. Catarrhal conjunctivitis	Bulls 1–6	Ocular swab	Negative	ND

ND: not done

According to data of the literature (Gourlay and Thomas, 1969; Langford and Leach, 1973; Goto et al., 1979; Schöttker-Wegner et al., 1990), all three isolated mycoplasmal species (*M. bovigenitalium, M. bovirhinis* and *M. bovoculi*) have been found in healthy bovine eyes as well as in association with bovine ocular disease. *M. bovigenitalium* and *M. bovirhinis* are frequently present in the bovine respiratory tract. While the pathogenicity of *M. bovirhinis* is uncertain, *M. bovigenitalium* is known to be pathogenic to the bovine udder and genital tract, and may be considered to be occasionally involved in bovine pneumonia (Gourlay and Howard, 1979). In our case these mycoplasmas were found in calves with conjunctivitis and respiratory disease. As conjunctivitis is not a consistent characteristic of infectious bovine rhinotracheitis, it may be considered an enhancing pathogenic effect of mycoplasmal infection on the appearance of this symptom. Our clinical observations confirm the opinion of Rosenbusch and Knudtson (1980) on the close association of *M. bovoculi* with IBK. From the epidemiological point of view, this paper presents the first report on findings of *M. bovigenitalium, M. bovirhinis* and *M. bovoculi* in bovine eyes in Croatia.

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DATA ON THE CONTAMINATION OF MAIZE WITH FUMONISIN B_1 AND OTHER FUSARIOTOXINS IN HUNGARY

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The fumonisin B_1, zearalenone, deoxynivalenol and T-2 toxin content of maize samples collected in the period of storage and harvesting was determined by high-performance liquid chromatography (HPLC) methods. Of the mouldy maize samples collected in the period of storage, 70.8% contained fumonisin B_1 (0.05–19.8 mg/kg; average concentration: 2.6 mg/kg), 87.5% contained zearalenone (0.01–11.8 mg/kg, average concentration: 1.26 mg/kg), 70% contained deoxynivalenol (0.07–21.2, average concentration: 4.33 mg/kg), and 41.7% contained T-2 toxin (0.06–0.39, average concentration: 0.2 mg/kg). These mycotoxins often occurred together in the samples. In the non-mouldy samples, both the positivity rate and the mycotoxin concentration were markedly lower. In the harvesting period, the mycotoxin content of maize ears more or less affected by moulds (so-called "affected sample") and of the average sample was determined separately for each maize-field involved in the study. Of the affected samples, 70% contained fumonisin B_1 (0.095–52.4 mg/kg; average: 6.64 mg/kg), 17% contained zearalenone (0.006–0.079 mg/kg; average: 0.03 mg/kg), 13% contained deoxynivalenol (0.05–0.118 mg/kg; average: 0.09 mg/kg), and 39% contained T-2 toxin (0.05–0.551 mg/kg; average: 0.165 mg/kg). Fumonisin B_1 and T-2 toxin often occurred together in the affected maize samples. Of the "average samples", 30% were contaminated with fumonisin B_1 (0.06–5.1 mg/kg; average: 1.52 mg/kg) and 9% with T-2 toxin (min.-max.-average: 0.05 mg/kg). The results call attention to the fact that maize cultivated in Hungary is often contaminated with fumonisin B_1. High concentrations of fumonisin B_1 were found primarily in the mouldy or affected maize ears; therefore, the development of diseases caused by fumonisin B_1 should be reckoned with primarily if such maize is fed. Besides fumonisin B_1, mouldy or affected maize usually contains also other fusariotoxins, which raises the possibility of mycotoxin interactions. Because of the high prevalence of fumonisin B_1 in maize grown in Hungary, the authors suggest that samples of maize used for feeding horses and pigs should be checked for fumonisin B_1 content.

Key words: Mycotoxin, fusariotoxin, fumonisin B_1, maize

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Fumonisins are mycotoxins produced by *Fusarium moniliforme*. They were first isolated from culture material of *F. moniliforme* strain MRC 826 grown on maize (Gelderblom et al., 1988) and, subsequently, their structure was elucidated (Bezuidenhout et al., 1988). The fumonisin group comprises six mycotoxins including fumonisin B_1, B_2, B_3, B_4, A_1 and A_2 (Cawood et al., 1991); however, according to the present state of knowledge fumonisin B_1 is the only member of the group which has animal health importance.

Fumonisin B_1 has been found to induce equine leukoencephalomalacia (ELEM; Marasas et al., 1988; Kellerman et al., 1990) and porcine pulmonary oedema (PPE; Harrison et al., 1990). Equine leukoencephalomalacia is a disease known for several decades, which is long known to be caused by the feeding of maize severely contaminated by *F. moniliforme* (Halibutron et al., 1979). Fumonisin B_1, the mycotoxin inducing the syndrome was, however, first isolated by South African researchers only a few years ago. Following the feeding of maize produced in the United States in 1989, mass cases of ELEM (Wilson et al., 1990) and PPE (Harrison et al., 1990) occurred. Both diseases were successfully reproduced with purified fumonisin B_1.

In toxicity tests, day-old birds of different poultry species including chickens, turkeys and ducks (Ledoux et al., 1992; Weibking et al., 1993) proved to be susceptible to fumonisin B_1, and so did cattle (Osweiler et al., 1993); however, natural disease caused by that mycotoxin has not been reported from these animal species so far.

In South Africa, the common maize contaminant fumonisin B_1 has been implicated in the aetiology of human oesophageal cancer which shows high prevalence in that area (Sydenham et al., 1990*b*).

The natural occurrence of fumonisin B_1 was first demonstrated in South Africa (Sydenham et al., 1990*a*). Subsequently, investigators regularly looked for the presence of that mycotoxin in feed samples analyzed in connection with cases of equine leukoencephalomalacia (Wilson et al., 1990; Binkerd et al., 1993). Fumonisin B_1 has also been detected in Europe, for the first time in Austria (cit. Visconti and Doko, 1994). Like strains isolated in South Africa and the United States (Thiel et al., 1991), the majority of *F. moniliforme* strains originating from European countries have also been found to produce fumonisin B_1 and B_2 (Visconti and Doko, 1994). Fumonisin B_1 often occurs in high concentrations in affected, mouldy ears of maize; however, in lower concentrations it may be present also in healthy maize samples. In addition to fumonisins present in high concentrations, mouldy maize often contains other fusariotoxins (zearalenone, deoxynivalenol, nivalenol) as well (Sydenham et al., 1990*b*).

Fusarium moniliforme, the fungal contaminant responsible for the moulding of maize ears, is highly prevalent also in Hungary (Palyusik, 1993). Animal disease caused by fumonisin B_1 and confirmed by the isolation of the mycotoxin has not been reported in Hungary yet. However, a subsequent analysis of earlier

descriptions of certain disease entities such as feed toxicosis in horses (Lacza and Lázár, 1977) raises the suspicion that these syndromes could have been caused by fumonisin B_1. In the 1950's, the so-called "fattening lung oedema" or characteristic pulmonary oedema frequently occurred in pigs. In the light of recent mycotoxin research, certain authors suggest that this disease could easily have been a mycotoxicosis caused by fumonisin B_1 (Kakuk, 1995). As fumonisin B_1 was likely to be present as a contaminant also on maize grown in Hungary, samples of maize produced in 1993 were assayed in the period of storage while those of maize harvested in 1994 were tested in the period of gathering in, using high-performance liquid chromatography (HPLC) methods, to determine their fumonisin B_1, zearalenone, deoxynivalenol and T-2 toxin content. Although the occurrence of fumonisin B_1 in maize cultivated in Hungary has recently been reported (Fazekas and Tóth Hajdu, 1995), maize may contain also other fusariotoxins which act in combination with fumonisin B_1 when the contaminated maize is fed. Therefore, in addition to the assay for fumonisin B_1, this study was extended to the determination of the most important other fusariotoxins as well. This paper reports the data obtained in the mycotoxin analyses.

Materials and methods

Maize samples

Maize samples collected in the period of storage. Fifty-two maize samples harvested in the autumn of 1993 were assayed. Sample collection took place in December 1993 and February 1994. Twenty-four samples were visibly mouldy and 28 were mould-free. Of the 24 mouldy samples, 21 had been stored in the ear and 3 as shelled kernels, while of the 28 non-mouldy samples 27 had come from a batch stored as shelled kernels and only one from a batch stored in the ear. Samples of maize stored in the ear had been derived from private producers in counties Heves and Pest, while those of maize stored as kernels had been obtained from feed mixing plants in Hajdú-Bihar County. The maize samples were inspected and their most important characteristics were recorded. Samples of maize in the ear were shelled, dried at 60 °C for 6 days and then stored, together with the grain samples, in deep freeze until assayed.

Maize samples collected in the harvesting period. The 23 samples of maize produced in 1994 were collected in the harvesting period from maize-fields in counties Pest, Heves and Hajdú-Bihar. Two samples were taken from each maize-field included in the study. One sample was taken from maize-ears more or less damaged by moulds (so-called "affected maize sample") while the other was

derived from 50 maize ears randomly selected on each maize-field (so-called "average sample"). The ears were shelled, then dried as described above and stored in deep freeze until assayed.

Mycotoxin assays

The determination of fumonisin B_1, zearalenone, deoxynivalenol (DON) and T-2 toxin was done by high-performance liquid chromatography (HPLC) methods. For the determination of fumonisin B_1, the samples were processed as described by Shephard et al. (1990). Extraction was done with a 3:1 mixture of methanol and water, and the extract was purified on a SAX SPE column. Samples derivatized with o-phthaldialdehyde (OPA) and mercaptoethanol were analyzed by HPLC. Sample processing for the quantitative determination of zearalenone (Csokán and Kovács, 1987), DON (Chang et al., 1984) and T-2 toxin (Rajakyla et al., 1987) was done as described previously. Instrumental analysis was carried out with a Hewlett-Packard 1050 type quaternary pump, HP 1050 type Diode Array Detector (DAD), and 1046 type fluorescence detector. For the separation procedure, LiChrospher RP 18, 250 × 4 mm columns of 5 μm particle size were used. The separation of fumonisin B_1 was carried out by isocratic elution of acetonitrile:water:acetic acid (50:50:1) composition (Stack and Eppley, 1992), while that of zearalenone and T-2 toxin by isocratic elution of acetonitrile:water (45:55) composition. For the separation of DON, gradient elution (0 min 100% water, 17 min 100% acetonitrile:water 20:80) was used. Fumonisin B_1 was detected in a fluorescence detector (excitation and emission wavelengths 335 and 440 nm, respectively), zearalenone simultaneously in a fluorescence detector (excitation and emission wavelengths 232 and 500 nm, respectively) and DAD (236 nm), while DON and T-2 toxin in DAD (218 and 194 nm, respectively). The pump and the detectors were controlled with the help of a 3D Chemstation software, and the chromatograms were evaluated. The identity of zearalenone, DON and T-2 toxin was confirmed also by the identity of the UV spectrum obtained in the DAD with that of the standard toxin. Quantitative determination was done with the help of a calibration curve.

The mycotoxin standards were obtained from Sigma, o-phthaldialdehyde and mercaptoethanol from Merck, HPLC-grade acetonitrile and methanol from Carlo-Erba, and the other chemicals from Reanal. The mycotoxin standards were dissolved in acetonitrile of HPLC grade and stored in deep freeze.

Results

Results obtained for samples collected in the period of storage

The chromatogram of fumonisin B_1 standard of 2 μg/ml concentration, obtained by HPLC analysis performed with the parameters described above, is presented in Fig. 1. Figure 2 shows the chromatogram of a maize sample positive for fumonisin B_1. The chromatographic peak (RT: 9.900) corresponds to 55 ng/20 μl fumonisin B_1, which represents a concentration of 15.3 mg/kg in the original sample. Figure 3 contains the chromatogram of a negative sample, with no interfering peak in the place of the fumonisin B_1 peak.

The ratio of mycotoxin-containing (positive) samples within the mouldy and mould-free samples is shown in Fig. 4. In the mouldy samples, fumonisin B_1, zearalenone and DON had a similar share in the positivity rate, while T-2 positivity was about half as high. In the mould-free samples, a much lower positivity rate was obtained for all the mycotoxins than in the mouldy samples.

The minimal, maximal and average mycotoxin values measured in the mouldy and mould-free samples are contained in Table 1. In the mouldy samples, DON was present in the highest average concentration, fumonisin B_1 and zearalenone occurred in somewhat lower concentrations, while the average concentration of T-2 toxin was about one order of magnitude lower. There were samples with expressly high (10–20 mg/kg) fumonisin B_1, zearalenone and/or DON content. At the same time, none of the samples showed a high level of T-2 contamination. In many of the samples analyzed, a co-occurrence of relatively high levels (some mg/kg) of fumonisin B_1, zearalenone and DON was found. The mycotoxin values measured in mould-free samples corresponded to contamination in traces.

Table 1

Mycotoxin content (mg/kg*) of mouldy and mould-free maize samples collected in the storage period

	Mouldy samples		Mould-free samples	
	Min.–max.	Average	Min.–max.	Average
Fumonisin B_1	0.05–19.8	2.6	0.05–0.151	0.1
Zearalenone	0.01–11.8	1.26	0.005–0.05	0.02
Deoxynivalenol	0.07–21.2	4.33	0.15–0.292	0.22
T-2 toxin	0.06–0.39	0.2	0.056–0.16	0.1

*Detection limit: fumonisin B_1: 0.05 mg/kg
zearalenone: 0.005 mg/kg
deoxynivalenol: 0.05 mg/kg
T-2 toxin: 0.05 mg/kg

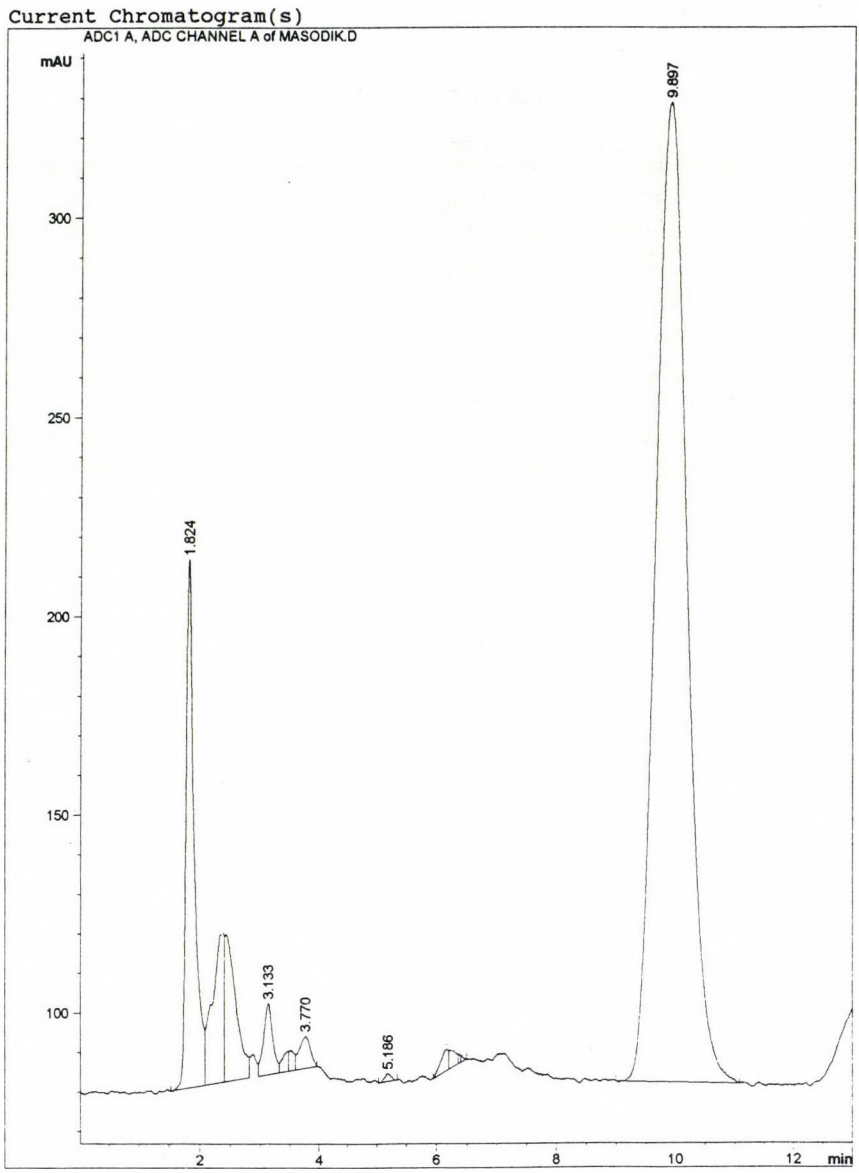


Fig. 1. Chromatogram (HPLC) of fumonisin B_1 standard (2 µg/ml), with fluorescence detection (RT: 9.897 min)

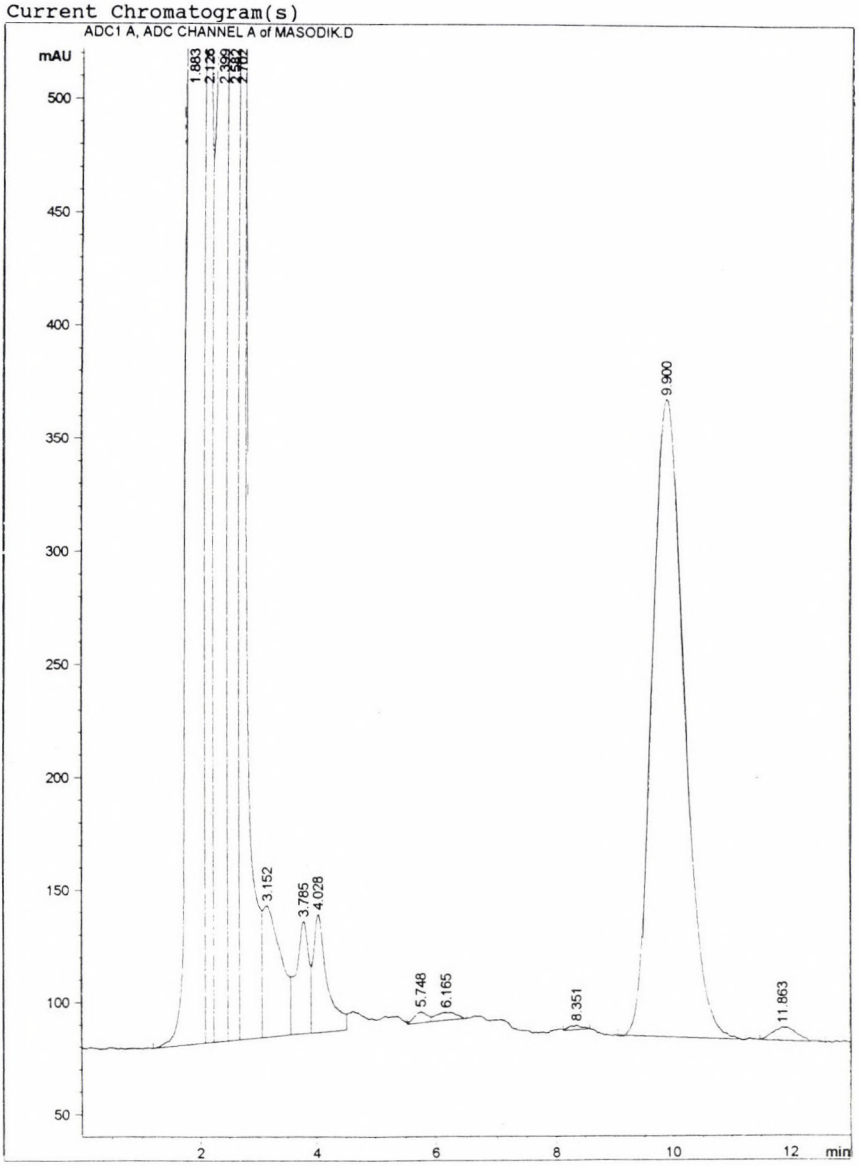


Fig. 2. Chromatogram of a fumonisin B_1 positive maize sample. The RT = 9.9 min peak corresponds to 55 ng/20 µl fumonisin B_1, which represents a 15.3 mg/kg concentration in the original sample

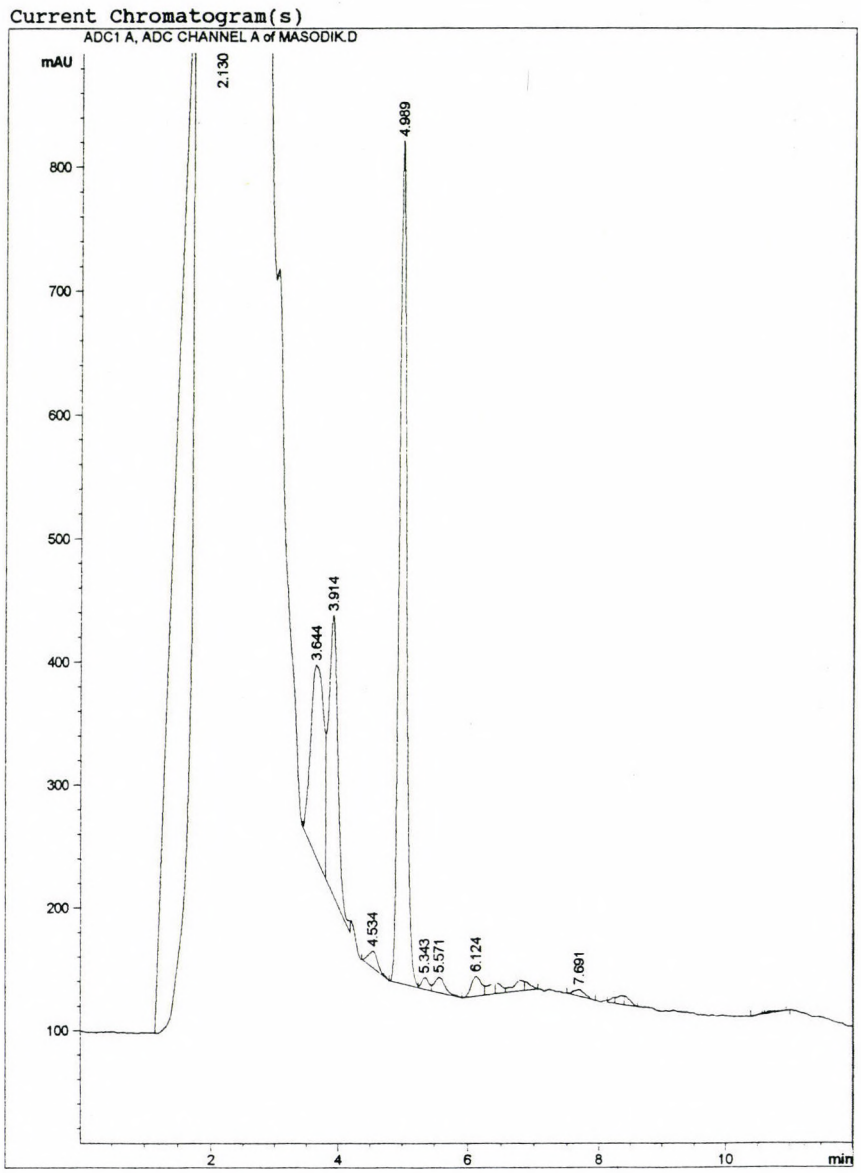


Fig. 3. Chromatogram of a negative maize sample. No interfering peak is seen in the place of the fumonisin B_1 peak of RT = 9.9 min

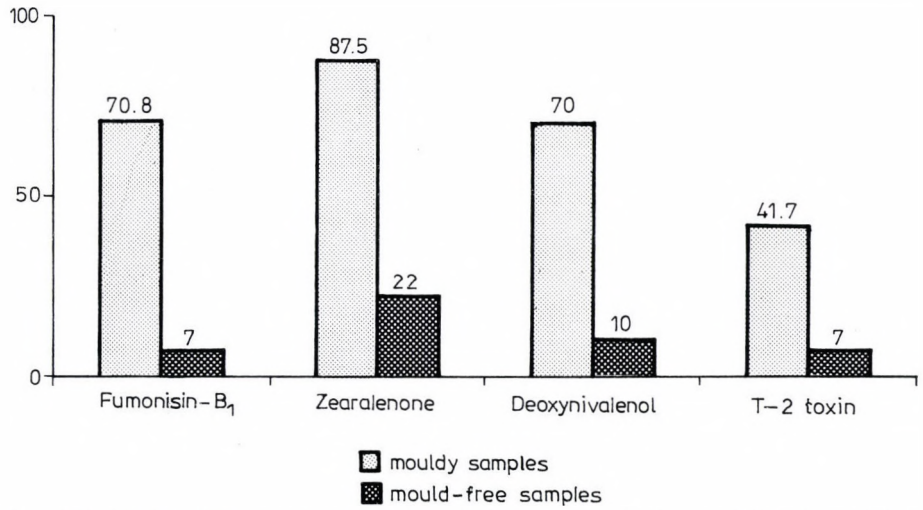


Fig. 4. Ratio (%) of mycotoxin-containing (positive) samples in mouldy and mould-free maize collected in the storage period

Results obtained for samples collected in the period of gathering in

The ratio of samples positive for the individual mycotoxins among the "affected" and the "average" samples is shown in Fig. 5. In the affected samples, fumonisin B_1 positivity was the highest and T-2 positivity was also relatively high, although only about half as high as the fumonisin B_1 positivity rate. Zearalenone and DON positivity was minimal. In the average samples, fumonisin B_1 was the only mycotoxin which occurred in a substantial ratio of the samples.

Table 2 presents the minimum, maximum and average mycotoxin levels measured in the "affected" and in the "average" samples. In many of the "affected" samples, fumonisin B_1 occurred in high concentrations which could be considered toxic to susceptible animal species (horse, pig). T-2 toxin often occurred together with fumonisin B_1, but in much lower concentrations. The quantitative ratio of the two toxins varied widely. There were samples in which the concentration of fumonisin B_1 was 100 times that of T-2 toxin; in other samples, the difference was only twofold. A certain part of the fumonisin B_1 samples did not contain T-2 toxin. Zearalenone and DON were measured in minimal quantities. Of the four mycotoxins studied, the "average" samples contained only fumonisin B_1. The highest level of fumonisin B_1 found in these samples was subtoxic for pigs and was at the lower limit of the toxic range for horses.

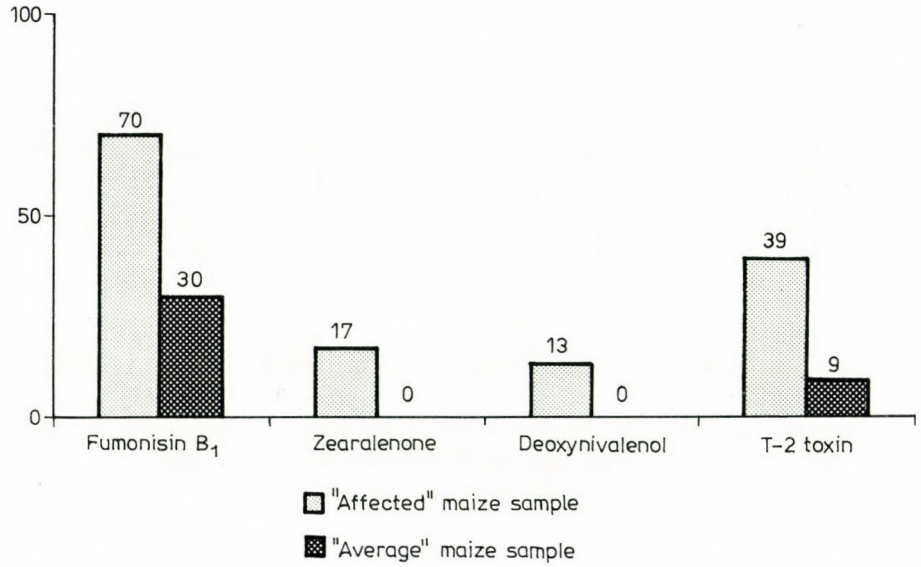


Fig. 5. Ratio (%) of mycotoxin-containing (positive) samples in the "affected" and "average" samples of maize collected in the harvesting period

Table 2

Mycotoxin content (mg/kg) of "affected" and "average" maize samples collected in the harvesting period

	"Affected" samples		"Average" samples	
	Min.–max.	Average	Min.–max.	Average
Fumonisin B_1	0.095–52.4	6.64	0.06–5.1	1.52
Zearalenone	0.006–0.079	0.03	0–0	0
Deoxynivalenol	0.05–0.118	0.09	0–0	0
T-2 toxin	0.05–0.551	0.165	0.05–0.05	0.05

Discussion

The results of this study indicate that fumonisin B_1 is a common contaminant of maize grown in Hungary, in the same way as has been reported for maize produced in the United States. High concentrations of fumonisin B_1 can be detected primarily in the mouldy or affected ears of maize; therefore, fumonisin toxicosis should be reckoned with primarily if ears and kernels of maize of high toxin content are fed in large quantities. The maize screenings (fines) separated during large-scale harvesting probably contain much higher concentrations of fumonisin

B_1 and, thus, can act as potential sources of fumonisin toxicosis. The fumonisin B_1 induced disease cases reported from the United States were often caused by the feeding of corn screenings (Ross, 1994).

The mycotoxin values measured in "average samples" collected in the harvesting period showed good agreement with the concentrations obtained in maize samples produced in Missouri (U.S.A.) in 1990 (Rottinghaus et al., 1992). In the latter study, a 15% fumonisin B_1 positivity was found, with concentrations ranging from 0.1 to 5 mg/kg, but other mycotoxins (aflatoxins, T-2 toxin, DAS, DON, zearalenone, etc.) were not detected. From other states of the U.S.A., e.g. Iowa, a fumonisin B_1 contamination of much higher prevalence and intensity was reported (Ross, 1994). In Hungary, contaminations of such high prevalence and concentration were found only in the expressly damaged or mouldy maize ears. The "average" samples typically contained fumonisin B_1 in subtoxic concentrations. Such concentrations of fumonisin B_1 are likely to cause mortality only rarely or not at all; however, they may easily impair the health status or performance of animals (e.g. reduce the body mass gain of pigs).

Comparing the results obtained for samples collected in the period of storage and harvesting, it was found that fumonisin B_1 is produced primarily in the vegetative period. This can be explained by the temperature and moisture requirement of fumonisin B_1 production. The optimum temperature for toxin production is 20 °C, while at temperatures below 8–10 °C toxin production practically ceases (Le Bars et al., 1994). At the same time, other fusariotoxins such as zearalenone and T-2 toxin require temperatures below 10 °C to be produced in high concentrations. This accounts for the observation that mouldy samples collected in the middle period of storage often contained, in addition to fumonisin B_1, also other fusariotoxins (zearalenone, DON) in high concentrations. On the other hand, affected maize ears collected in the harvesting period contained only fumonisin B_1 in high concentrations, obviously because the temperature conditions of the vegetative period had been unfavourable for the mass production of the other fusariotoxins. It was striking that affected maize ears collected in the harvesting period often contained T-2 toxin parallel to fumonisin B_1, even if only in relatively low concentrations. As far as we know, this is the first report on the co-occurrence of fumonisin B_1 and T-2 toxin. It would be interesting to know whether the co-occurrence of these two mycotoxins gives rise to a toxic interaction in susceptible animal species. Studies are currently under way to answer this question.

The results of this survey covering several years show that fumonisin B_1 is a common contaminant of maize also in Hungary. This poses the potential risk of mortality or other health problems in susceptible animal species. Fumonisin B_1 has carcinogenic potential in humans and, thus, it is of great public health importance. In animals, the deleterious effects of fumonisin B_1 can be prevented by avoiding the feeding of maize which is known to contain high levels of that toxin.

For this, the fumonisin B_1 content of maize lots must be determined. Therefore, it is advisable to regularly check the fumonisin B_1 content of maize lots used for feeding horses and pigs.

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FACTORS INFLUENCING MICROBIAL GROWTH AND THE EFFICIENCY OF MICROBIAL PROTEIN SYNTHESIS: A REVIEW

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The possibilities of influencing microbial growth and the efficiency of microbial protein synthesis are reviewed. The authors present a detailed discussion of the effects exerted by the level of feed intake, the feeding frequency, the concentrate to forage ratio, the quality of the carbohydrate and protein source, and the interaction of carbohydrate and protein on microbial protein synthesis and, consequently, on the flow of microbial protein. An in-depth knowledge of the above factors influencing bacterial growth and microbial fermentation is essential for the well-balanced feeding of ruminants (primarily dairy cows) of high production level.

Key words: Microbial growth and efficiency, carbohydrates, proteins, feed, rumen, degradability

The microbial community constituting the microbial ecosystem of the rumen and consisting of obligate and, to a smaller extent, facultative anaerobes contains 10^9 to 10^{11} microorganisms per millilitre. The composition and distribution of that microbial community vary depending on the feed which acts as substrate. The digestible organic matter of the feed acts as substrate from which the rumen microflora and microfauna form organic acids and ammonia while generating a considerable amount of microbial mass. The most important factor that limits bacterial growth is the energy generated during the fermentation of carbohydrates into organic acids and available in the rumen.

Bauchop and Elsden (1960) were the first to study the relationship between microbial biomass production and energy supply. They introduced the concept marked as Y_{ATP}, which shows how many grams of bacterial dry matter can be synthesized from one mole of ATP (g of bacterial dry matter / moles of ATP). According to their model calculations, 3.62 moles of ATP are needed for the synthesis of 100 g of bacterial dry matter (i.e., 28 g can be synthesized from 1 mole of ATP). However, as shown in Fig. 1, that value cannot be reached, only approximated, under practical conditions. According to the above-mentioned authors, the

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values are between 8.3 and 12.5 g/mole of ATP, which means that, in general, one mole of ATP can give rise to 10.5 g dry matter of bacterial origin. For a long time this had been regarded as a constant value until in subsequent studies higher values were measured. For instance, Baldwin (1970) reported the formation of 16.5 g bacterial dry matter/mole of ATP, while for certain bacteria Russell and Wallace (1989) found even higher values (around 20 g).

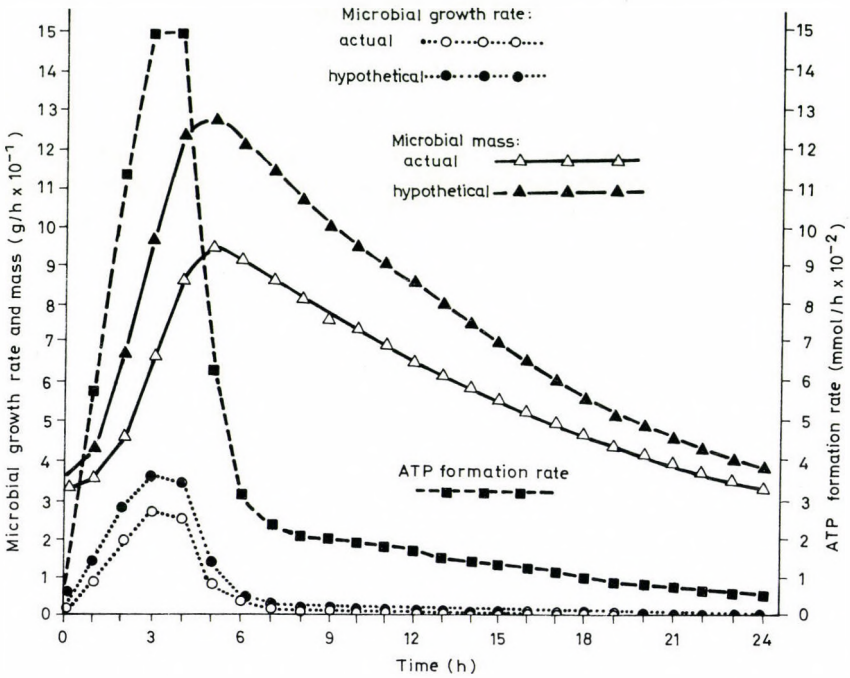


Fig. 1. Rate of ATP formation, microbial growth rate and rumen microbial mass under hypothetical maximal conditions and actual conditions used in the model under once daily feeding (Baldwin and Denham, 1979)

Within the energy, the quantity of generated bacterial dry matter greatly depends on the source from which it arises. Namely, this exerts a decisive influence on the microbial growth rate. From Fig. 2 it appears that microorganisms show the lowest growth rate if cellulose is used as energy source, while the highest growth rate is obtained with monosaccharides as a source of energy. The lowest growth rate results in the formation of a smaller bacterial mass. Curves A, B and C of Fig. 2 indicate that the formation of bacterial mass is influenced also by the parameter marked with M, i.e. the energy used for the maintenance of microorganisms (Pirt, 1965). The so-called maintenance function of bacteria is not fully clear; however, the maintenance of ion balance across the cell membrane is prob-

ably the most important factor in this respect (Tempest and Neijssel, 1984). Like the growth of animals, bacterial growth cannot occur until the maintenance requirement is met. If the growth rate is low, the bigger part of the total energy consumption is used for the maintenance of the cell.

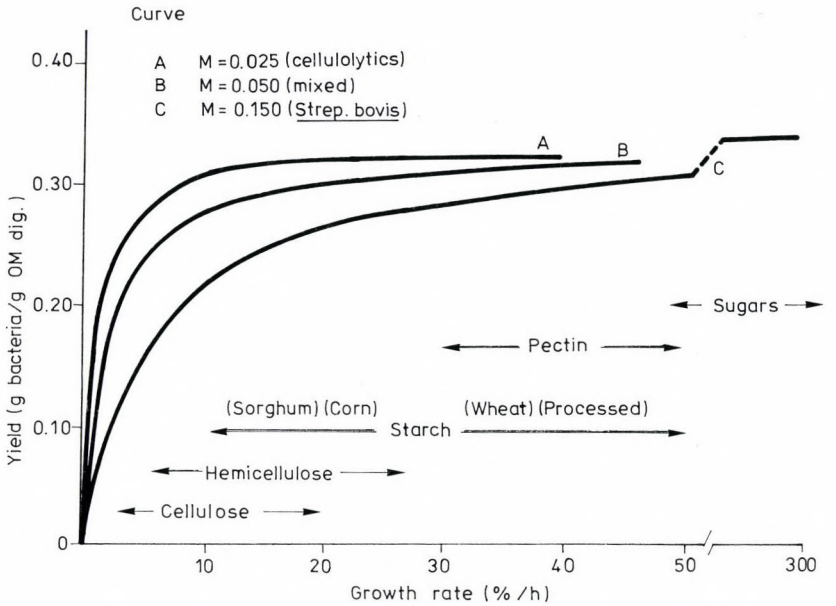


Fig. 2. Effect of microbial growth rate on the yield of rumen bacteria from various feed components (Sniffen and Robinson, 1987)

The quantity of generated microbial mass which passes from the rumen into the duodenum (microbial yield) can be calculated from the energy requirement, the maintenance requirement and the growth rate. The relationship of these parameters is expressed by the following equation (Nocek and Russell, 1988):

$$1/Y = m/k + 1/Yg,$$

where

Y (yield) = g bacteria / g fermented carbohydrate;
m (maintenance) = g carbohydrate / g bacteria / h;
k = growth rate / h;
Yg = the theoretical maximum growth yield at a given maintenance level (g bacteria / g carbohydrate).

Daily microbial protein yield is the product of the microbial efficiency. This latter variable shows how many grams of microbial N or protein have been formed

per one kg of organic matter apparently digested (ADOM) or organic matter truly digested (TDOM). Calculating the average of 46 determinations (30 performed in sheep and 16 in cattle), Stern and Hoover (1979) reported a value of 27 g microbial N/kg of TDOM (16.9 g crude protein/100 g TDOM).

From the above it appears that the growth rate of microorganisms, the efficiency of microbial protein synthesis and, thus, the daily microbial protein yield are closely related. Therefore, the factors described below affect both the growth of microorganisms and the microbial yield.

This review discusses the following factors influencing the efficiency of microbial protein synthesis: the level of feed intake, the feeding frequency, the concentrate to forage ratio, the carbohydrate source, the protein source and, finally, the interaction of carbohydrate and protein.

Level of feed intake

When the level of feed intake was increased, a higher flow of microbial N from the rumen was measured in numerous experiments (Greife et al., 1985; Robinson et al., 1985; Merchen et al., 1986). In other studies (Chamberlain et al., 1976; Tamminga et al., 1979; Firkins et al., 1984), however, no correlation was found between the feed intake and the microbial yield. This may have been due to the narrow range of feed intake levels used (between 1% and 2.5% of the body weight) in the above-cited studies carried out in sheep and in beef cattle, respectively. According to the results obtained by Tamminga (1981) and Robinson (1983), in dairy cows a clear-cut close correlation could be established between the feed intake and the microbial yield (Fig. 3). Tamminga's experiments included a total of 43 dairy cows fed a ration containing 47% hay. The lowest microbial yield was measured at a dry matter intake of 10 kg (1.8% of the body weight). Intakes above and below 10 kg both resulted in an increased yield.

Van Soest (1982) suggested that a higher intake results in a greater flow of particles from the rumen (Fig. 4) and, thus, more bacteria adhering to feed particles will pass into the duodenum. As a result, the higher flow of dietary organic matter will decrease the microbial recycling. It can be seen from Fig. 4 that the turnover rate of not only the solid but also the liquid phase of the rumen content is in a positive correlation with the feed intake (Evans, 1981). The flow rate of the liquid phase increases parallel to the increase in feed intake. The higher microbial yield found in such cases is probably a consequence of the enhanced "wash-out" of microorganisms from the rumen (Leng et al., 1984). Attempts to achieve a further increase in the microbial yield by raising the outflow rate of rumen fluid above a certain level (4–6%/h) failed (Fig. 5). In many cases, increasing the rumen liquid

dilution rate caused the opposite effect, i.e. a reduction in the microbial yield (Chamberlain and Thomas, 1980; Hadjipanayiotou et al., 1982; Goetsch and Owens, 1985).

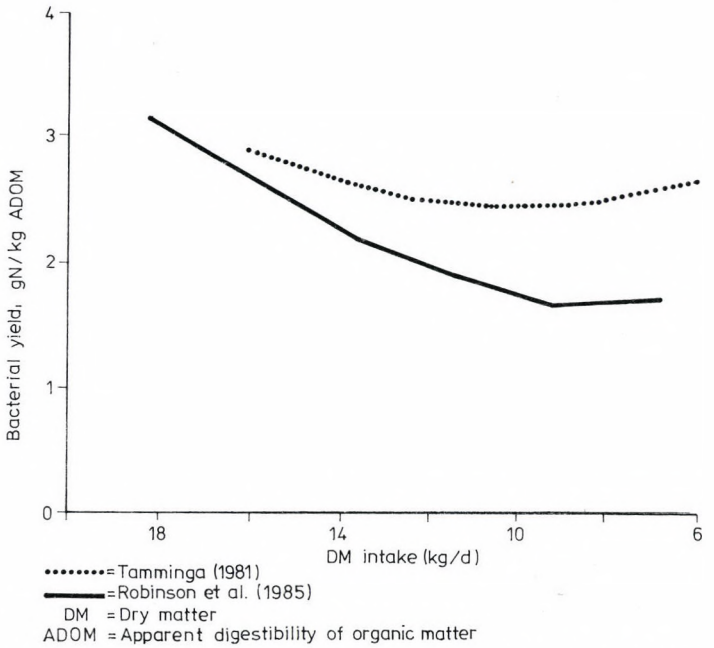


Fig. 3. Relationship between bacterial yield and declining feed intake in cows fed high forage diets (Sniffen and Robinson, 1987)

Feeding frequency

Only few researchers have studied the relationship between microbial flow and the feeding frequency, even though this appears to be the most feasible way of influencing the microbial yield. The available results are rather contradictory. This is surprising, as it would seem logical to expect that by increasing the feeding frequency diurnal fluctuations in the quantity of metabolites derived from microbial fermentation will be avoided and, through the more balanced rumen fermentation, microbial yield can be increased. The results obtained by Tamminga (1981) support this hypothesis. As compared to twice daily feeding, six feedings per day resulted in an increased microbial N flow and an enhanced ruminal true digestibility of organic matter (TDOM). Other investigators (Beever et al., 1972; Brandt et al., 1981; Robinson and Sniffen, 1985) either did not find any increase in microbial flow as a result of more frequent feeding, or — as opposed to Tamminga's findings — they observed a higher bacterial yield (Robinson, 1983) and protozoal

yield (John and Ulyatt, 1984) with less frequent feeding. Sniffen and Robinson (1987) attribute the higher microbial yield measured in the case of less frequent feeding to the rapid feed intake over a short period and to the resulting higher outflow rate of the rumen content toward the duodenum. The higher flow rate increases the outflow of bacteria adhering to feed particles and thus decreases the bacterial recycling. This latter leads to enhanced microbial growth. The higher protozoal yield is attributed to the fact that after feeding, because of the increased soluble substrate, the number of protozoa will increase in the liquid phase of the rumen content, and the increased ruminal outflow rate will lead to an enhanced "wash-out" of protozoa.

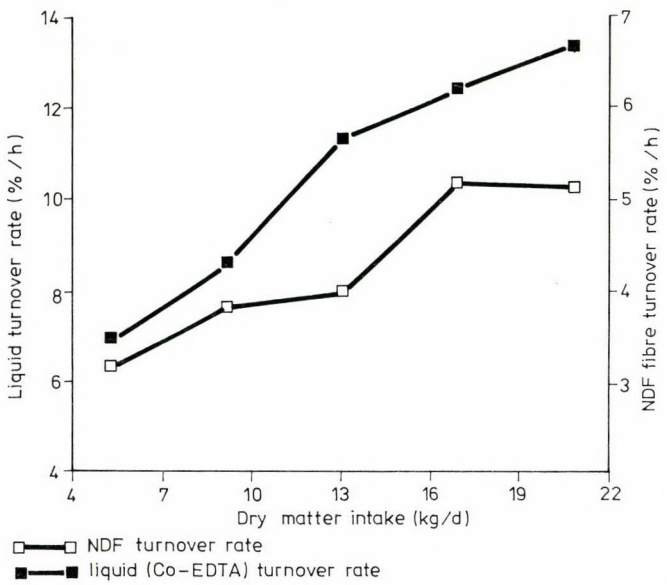


Fig. 4. Relationship between feed intake and turnover rate of liquid and NDF fractions of digesta (Sniffen and Robinson, 1987)

Concentrate to forage ratio

Several studies have indicated that the rising forage content of the ration results in a higher microbial yield (Fig. 6). In sheep, the maximum bacterial yield was measured if the forage intake constituted 70% of the ration. Forage intakes below, but especially those above that level were found to result in a lower yield (Chamberlain and Thomas, 1979; Mathers and Miller, 1981). After increasing the forage level of the ration from 0% to 25% and from 10% to 40% in steers and dairy cows, respectively, a higher microbial yield was measured (Cole et al., 1976;

Oldham et al., 1979). The report issued by the NRC (1985) also seems to support the observation that diets containing less than 40% forage (20% NDF) resulted in a decreased microbial growth yield. From the results of Russell et al. (1992) it can be established that at an NDF content lower than 20% of the DM, microbial yield is reduced by 2.5% for every 1% decrease in NDF. The reduction observed in yield at a higher concentrate level of the ration is explained by an uncoupled fermentation. Namely, at a higher forage level the slower degradation of organic matter allows more energy to be trapped by microorganisms. In the studies cited above, the concentrate contained mainly barley and corn grains. This must be emphasized because no correlation was found between the concentrate to forage ratio and the microbial yield if the concentrates were composed of high-fibre by-products (Tamminga, 1981).

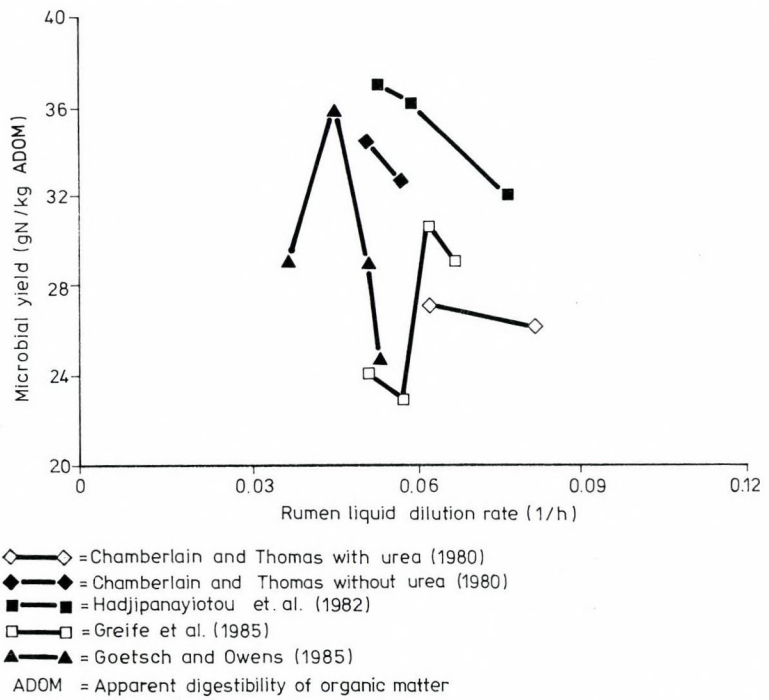


Fig. 5. Relationship between rumen liquid dilution rate and microbial N yield (Sniffen and Robinson, 1987)

A positive correlation was established between the proportion of forage in the diet and the rumen liquid turnover rate (Evans, 1981). At the same time, at a forage level exceeding 70–75% a reduction was observed in microbial yield. In that case the lower washout of microbial matter and higher bacterial recycling in the rumen diminish the growth of bacteria, and the greater proportion of energy is

diverted for maintenance. This must have accounted for the observations of Chamberlain and Thomas (1979) and Mathers and Miller (1981), who measured a low microbial yield with an all-forage diet.

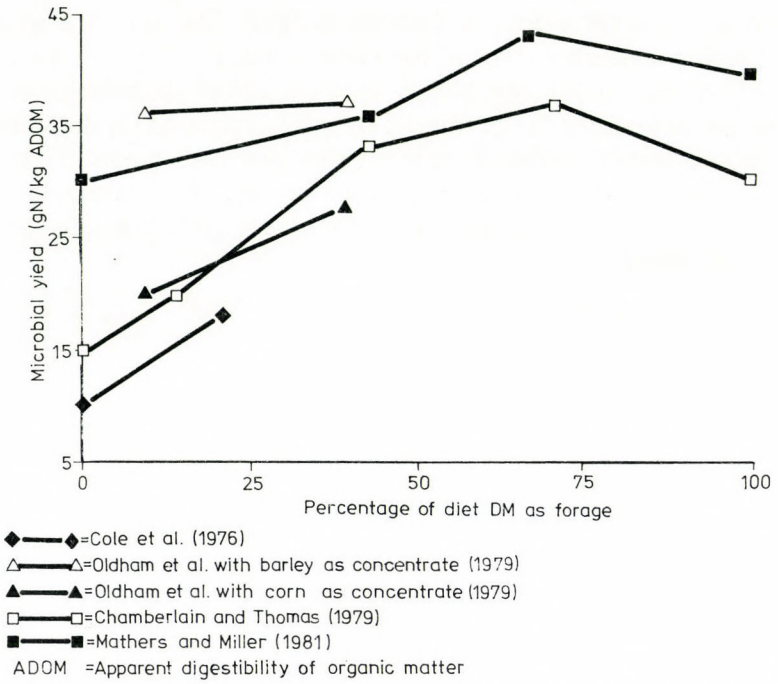


Fig. 6. Relationship between proportion of the dietary dry matter (DM) intake as forage and microbial yield (Sniffen and Robinson, 1987)

The effect of the concentrate to forage ratio may be markedly modified by the level of feed intake. Studying the relationship of these two variables, Merchen et al. (1986) found that at a lower feed intake there was no difference in the efficiency of microbial protein synthesis between rations containing 75% or 25% concentrate. In contrast to this, at a high feed intake the ration containing 75% concentrate was found to result in a higher microbial N outflow and to improve microbial efficiency. The complexity of the fermentation processes taking place in the rumen is shown by the fact that, as opposed to what has been described above, the above-cited authors explain the increased duodenal flow of bacterial N and the enhanced efficiency of microbial protein synthesis by the larger amount of starch (easily fermentable carbohydrate) present in diets high in concentrates. This reasoning is supported by the *in vitro* study of Stern et al. (1978) who, stressing the importance of an exact knowledge of feed constituents, experimentally confirmed

that the efficiency of microbial protein synthesis increases when the nonstructural carbohydrate (NSC) content of the ration is raised. Further details of this problem will be given in the following section.

Carbohydrate source

Like animal cells, the cells of plants are constituted by nitrogenous compounds, carbohydrates, lipids and minerals. In the composition of carbohydrates, however, there is a major difference, since the cell wall of plants contains molecules which cannot be found in the animal cell.

The structure of the plant cell (Patton, 1994) is shown below.

<table>
<tr><td rowspan="3">Constituents of plant cells</td><td>Ash</td><td></td><td></td></tr>
<tr><td>Lipids</td><td></td><td></td></tr>
<tr><td>Nitrogenous compounds</td><td></td><td></td></tr>
<tr><td rowspan="2">"Sugar"</td><td rowspan="5">Total carbohydrates</td><td rowspan="2">Nonstructural carbohydrate (NSC)</td></tr>
<tr><td rowspan="2">Starch</td></tr>
<tr><td rowspan="4">Constituents of plant cell wall</td><td>Pectin</td><td rowspan="4">Structural carbohydrate (SC)</td></tr>
<tr><td>Hemicellulose</td></tr>
<tr><td>Cellulose</td></tr>
<tr><td>Lignin</td></tr>
<tr><td>Ash</td><td></td><td></td></tr>
</table>

Ash: total mineral substances
Lipids: triglyceride, fatty acids, vitamins A, D, E and K, sterols, pigments
Nitrogenous compounds: ammonia, urea, purine, pyrimidine, amino acids, total unbound proteins
Simple sugars and their derivatives: glucose, fructose, arabinose, glucuronic acid, galactose, ribose, xylose, galacturonic acid
Starch: amylose and amylopectin
Pectin: polymer of methyl-D-galacturonate with α 1-4 linkages
Hemicellulose: xylan formed from D-xylose with β 1–4 linkages
Lignin: polymerized aromatic alcohols
Cellulose: polysaccharide with β 1–4 linkages, composed of D-glucose units.

The carbohydrates occurring in plants can be assigned to two main groups: nonstructural carbohydrates including e.g. sugars and starch, and structural cell-wall constituent carbohydrates such as pectin, cellulose and hemicellulose.

The ruminal utilization of carbohydrates is decisively influenced by the ease of solubilization. Correspondingly, sugar, starch and pectin are the carbohydrates most rapidly fermented. When feeding a diet of 77:23% forage to concentrate, the quantity of starch and simple sugars in the rumen decreased to a minimal level by 8 h postfeeding (Leedle et al., 1986). With diets of a higher concentrate ratio (68%), the degradation of carbohydrates took place in a manner different from that described above. Starch was fermented rapidly also in this case, but the sugar level first increased and it started to decrease only 4 h after the feed intake. After its initial decrease, the pectin content of the rumen remained constant for 24 h, which the authors explained by the concurrent pectin degradation and galacturonic acid utilization.

Of the structural carbohydrates, hemicellulose was fermented more rapidly than cellulose, as indicated by the hardly decreasing cellulose content until 12 h postfeeding (Leedle et al., 1986). In contrast, in their experiment Glenn and Canale (1990) measured a lower ruminal digestion of hemicellulose and xylose than that of cellulose and glucose. The slower rate of hemicellulose hydrolysis is supported by the observation that xylan, the major constituent of hemicellulose, produced the lowest yield, whereas cellobiose produced the highest (Strobel and Russell, 1986).

The degradation of carbohydrates may be modified by the pH value of the rumen fluid. At a constant pH value (6.7) the efficiency of microbial dry matter production (Y_{ATP}) during the degradation of starch, sugars, cellobiose, xylan and pectin was similar (Strobel and Russell, 1986). Lower pH markedly decreased the digestion of cellulose, hemicellulose and pectin while it only slightly affected that of starch (Marounek and Bartos, 1983; Hoover, 1986; Shriver et al., 1986; Ben-Ghedalia et al., 1989). According to certain studies (Russell and Dombrowski, 1980; Finlayson, 1986), the value of Y_{ATP} markedly decreases in the pH range between 6.5 and 5.5. As compared to the level measured at pH 6.7, Strobel and Russell (1986) observed a 50% decrease in the efficiency of microbial protein synthesis at pH 5.7. At the same time, in other studies it was found that the decrease of pH did not affect or even increased the microbial efficiency, measured as Y_{DOM} (digestible OM) (Hoover et al., 1984; Shriver et al., 1986).

The rate of degradation is influenced also by the chemical structure of the carbohydrate concerned. The degradation rate of plant cell wall decreases if the structural carbohydrates contain large quantities of lignin, acetal and phenol ester (Hespell, 1988). Despite their simpler chemical structure, nonstructural carbohydrates differ in their ruminal degradation, and this influences the total microbial protein yield per day. The starch present in certain plants (oats, tapioca, barley) is degraded 2 to 2.8 times as fast as that contained in corn (Cone et al., 1989). Certain treatments of the feed (e.g. extrusion) may double the digestion of starch. The efficiency of microbial protein synthesis (g N/kg ADOM) was higher in dairy cows fed barley grains than in those fed corn (Oldham et al., 1979). This effect

was even more expressed if poor-quality hay (containing 6.4% crude protein) was fed as basal feed. The feeding of pelleted rye-grass hay supplemented with barley resulted in a markedly higher microbial yield than if the same hay was supplemented with corn (Voight et al., 1977). On the other hand, no difference was found between barley and corn supplementation if the hay was fed in chopped form. Pelleting facilitates the access of bacteria to fibre and, thus, improves the ruminal degradation of hay. The barley starch added to the hay provides a rapidly degraded source of energy to support microbial growth. In contrast to the above-cited study, sorghum grain, barley and corn did not change the efficiency of microbial protein synthesis if the animals were fed a ration containing 20% forage (Spicer et al., 1986).

Protein source

Rumen bacteria can utilize N-containing substances of different origin for protein synthesis. In most cases, ammonia acts as the principal nitrogen source. Depending on the carbohydrate source from which they obtain the energy necessary for their growth, bacteria markedly differ also in terms of N utilization. Thus, bacteria fermenting structural carbohydrates (cellulose, hemicellulose) utilize exclusively ammonia (Bryant, 1973), whereas those capable of degrading nonstructural polysaccharides (NSC) use either ammonia or amino acids and peptides for the protein synthesis (Russell et al., 1992). According to the results of *in vitro* studies, 66% of the NSC microbial protein is derived from peptides or amino acids, and 34% comes from ammonia (Russell et al., 1983). This ratio is not affected by the growth rate of bacteria but is markedly influenced by the available energy. Namely, in the absence of easily fermentable carbohydrates the bacteria are unable to incorporate peptides which thus will be degraded to yield ammonia.

Bacteria utilize the products of proteolysis with dissimilar efficiency. Large peptides are taken up more rapidly and are used more efficiently for microbial growth than amino acids and small peptides (Prins et al., 1979; Chen et al., 1987; Broderick et al., 1988; Wallace et al., 1990). As compared to peptides of a molecular weight less than 1500, for peptides with a molecular weight exceeding 10,000 an increase in microbial growth was observed (Thomsen, 1985).

The microbial growth may be influenced by the N sources. Compared with ammonia, amino acids improved microbial efficiency, measured as weight microbial N/weight carbohydrate digested (Maeng and Baldwin, 1976). Higher microbial growth yield was obtained if amino acids and peptides were used in combination than if their effect was studied separately (Argyle and Baldwin, 1989). Still, complete proteins of high degradability (e.g. casein, soybean) proved to be the most efficient substrates. In their presence the digestion of starch doubled and the degradation of cellulose also increased considerably (Hugue and Thomsen, 1984; Thomsen, 1985). Despite the observations cited above, ammonia represents the

principal nitrogen source for microbial protein synthesis and, therefore, its importance must not be underestimated. At a low ammonia level the growth of obligate ammonia-utilizing bacteria (e.g. cellulolytics) is lower, which results in a decreased carbohydrate degradation and reduced feed intake (Tamminga, 1980). Protein sources resistant to ruminal degradation (fish meal, blood meal) resulted in a decreased acetate to propionate ratio and produced a lower ruminal ammonia concentration (Fébel et al., 1993; Kellems et al., 1993).

Opinions vary as to the optimum ammonia concentration necessary for adequate microbial synthesis: the reported values are between 2.9 and 13.5 mmol/l (5 and 23 mg/100 ml). According to Satter and Slyter (1974), maximum microbial protein synthesis can be achieved already at an ammonia level as low as 2.9 mmol/l (5 mg/100 ml). Mehrez et al. (1977) observed the same at a much higher ammonia concentration (11.2–13.5 mmol/l = 19–23 mg/100 ml). From the results reported by Hespell and Bryant (1979) it can be established that a higher ammonia level is required for maintaining maximum digestion and fermentation rates if the ration contains a large quantity of concentrate (large amounts of rapidly degraded carbohydrate). Erdman et al. (1986) established the following linear regression equation for the calculation of optimum ammonia level: NH_3N (mg/100 ml) = 0.452 × dry matter fermentability – 15.71 ($R^2 = 0.50$).

The ammonia concentration of the ruminal fluid markedly influences the nitrogen assimilation, too (Leng and Nolan, 1984). At a high ammonia level glutamate dehydrogenase while at a low ammonia content glutamate synthetase and glutamine synthetase take part in ammonia fixation. Glutamine synthetase requires ATP for its activity; thus, in the absence of sufficient energy, 14% less microbial yield will be obtained.

Several studies have confirmed that urea supplementation to a low, or no, N diet results in increased microbial flow. Such an increase was obtained only up to a certain urea level above which a reduction was noted (Kang-Meznarich and Broderick, 1981). Other authors are of the opinion that the microbial yield cannot be increased further by incremental urea supplementation (Hume et al., 1970; Leibholz, 1980). Parallel to increasing the dietary amount of a very rapidly degraded source of nitrogen (e.g. alfalfa protein), microbial yield will first increase then drastically decrease (Veira et al., 1980; Lu et al., 1982; Veira and Ivan, 1982). The efficiency of protein synthesis was found to decrease if a urea-N or rapidly degradable N supplementation exceeding 25 g N/kg of dry matter intake was provided. In lactation rations the N intake of high-yielding dairy cows (producing over 30 kg of milk per day) is usually higher than that. Tamminga et al. (1979) set the critical value at 39 g N/kg of dry matter intake; however, in their experiments the protein sources used were of moderate solubility.

The interaction of carbohydrate and protein

During proteolysis it often occurs that the ammonia production rate exceeds the utilization rate. Absorbed from the rumen, this ammonia can no longer be utilized by the microbes: some of it is transformed into urea and excreted in the urine. By the use of ^{15}N, Nolan (1975) found that in this way as much as 25% of the protein N may be lost for the bacteria and, eventually, the host. Carbohydrates have little effect on the rate of protein degradation by extracellular proteinases. At the same time, the further fate of amino acids arising during proteolysis is markedly affected by the quantity of fermentable carbohydrate available. If sufficient ATP is present, amino acids entering the microbes can be incorporated into microbial protein. NSC bacteria take up peptides at a rate of 0.07 g peptide/g bacteria/h. This N is used for microbial protein synthesis or ammonia production (Russell and Martin, 1984; Hino and Russell, 1985). If ATP is not sufficient to drive protein synthesis, amino acids will be fermented as an energy source and ammonia will accumulate. Thus, eventually the diversion of peptides to microbial protein or ammonia is regulated by the availability of carbohydrate.

The ruminal degradation of carbohydrate is thus one of the major factors influencing the microbial protein yield. Carbohydrate degradation is positively influenced by the rising nonstructural carbohydrate (NSC) content of total dietary carbohydrate and the increasing quantity of degradable intake protein (DIP); however, NSC has a more expressed effect in this respect (Hoover and Stokes, 1991). The efficiency of microbial protein synthesis increases when the NSC and DIP level of the diet is raised, but at a dissimilar rate. In *in vitro* studies, the efficiency of microbial protein synthesis increased, irrespective of the DIP, if the NSC content was raised from 25% to 37%. At the same time, no further improvement was obtained by raising the dietary NSC content from 37% to 54%, which indicates that optimum NSC content must be around 37% of the dry matter (Stokes et al., 1991*b*). This assertion is at variance with the results reported by Chamberlain and Thomas (1979), Oldham et al. (1979), and Mathers and Miller (1981), who are of the opinion that the efficiency of microbial protein synthesis will decrease if the concentrate level of the ration exceeds 30%. In their experiments the DIP remained constant when the concentrate intake and thus the NSC content were increased, which meant a wider NSC:DIP ratio. If the ratio of protein and carbohydrate is inadequate, microbial growth will be limited due to energetic uncoupling. In their above-cited experiment, Stokes et al. (1991*b*) found that a decrease of the DIP, i.e. the increase of the NSC:DIP ratio impaired the efficiency of microbial protein synthesis on all three levels of NSC used (Fig. 7). The results obtained by Varga et al. (1988) also stress the importance of having an accurate knowledge of the NSC:DIP ratio. At NSC:DIP ratios higher than 6:1 they observed a decrease

in microbial growth as well as in the degradation of fibre and protein. When the NSC:DIP ratio was decreased (to 3.4:1), the efficiency of microbial protein synthesis increased.

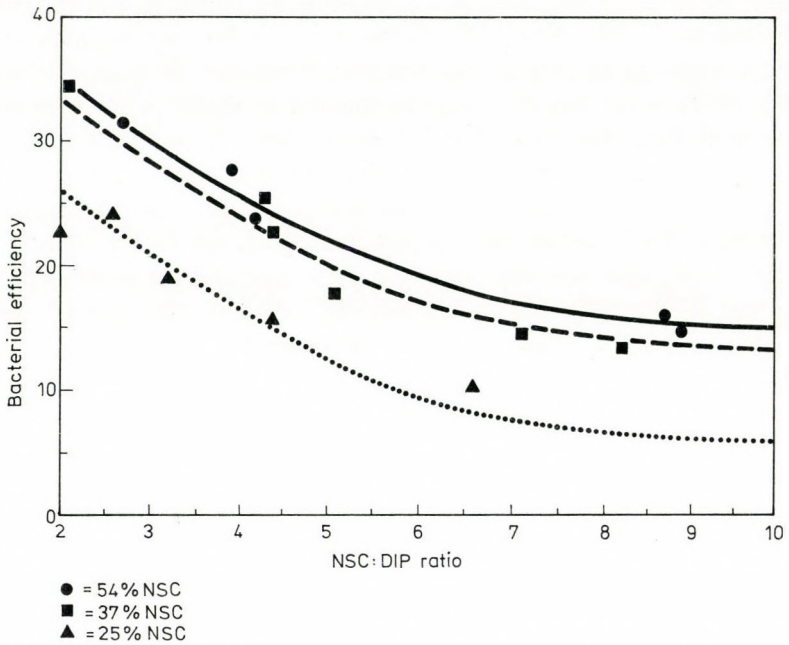


Fig. 7. Bacterial efficiency (g of bacterial N/kg of DM digested) in relation to nonstructural carbohydrate:degradable intake protein (NSC:DIP) ratios (Stokes et al., 1991*b*)

Stokes et al. (1991*a*) conducted a trial with dairy cows provided with rumen and duodenal cannulas. Their experiment provided further valuable data on the relationship between the NSC:DIP ratio and the efficiency of microbial protein synthesis. They studied the effects of a diet containing 38, 31 or 24% NSC and 13.2, 11.8 or 9% DIP, respectively. Parallel to the decrease of NSC and DIP, the ruminal degradation of organic matter, carbohydrate and protein decreased and the content of microbial N in the duodenum became lower. At the same time, the results of *in vivo* experiments (Stokes et al., 1991*a*; Fébel et al., 1994) indicate that, despite the low NSC and DIP content of the ration (24% NSC and 9% DIP), the efficiency of microbial protein synthesis was not impaired if the NSC and DIP were decreased proportionately (low NSC:DIP ratio).

Evaluating the results of experiments on the effects of dietary NSC and DIP, it can be established that in the ration of dairy cows a nonstructural carbohy-

drate level of 31–39 % NSC per kg of dry matter should be recommended as a target to be reached. As regards the rumen degradable intake protein requirement, the ration of high-yielding dairy cows must contain at least 11% DIP of DM.

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COMPLEX STUDY OF THE PHYSIOLOGICAL ROLE OF CADMIUM IV. EFFECTS OF PROLONGED DIETARY EXPOSURE OF BROILER CHICKENS TO CADMIUM

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A 274-day long cadmium (Cd) feeding trial was carried out with broiler cockerel chickens. The diet of the control group (Cd-0, n = 6) contained no added Cd, whereas to the diet of group Cd-25 and group Cd-75 (n = 10 each) 25 ppm and 75 ppm Cd was added, respectively, in the form of $CdSO_4$. The chickens were subjected to examinations described in detail earlier (Bokori et al., 1995*b*). In addition, the relative mass of five different organs (heart, liver, testis, spleen and brain), expressed as % of the body mass, was also determined. The clinical, gross and histopathological examinations and the assay of organs for mineral element content led to the following main findings. The feeding of diets containing 25 or 75 ppm Cd for more than 9 months did not cause signs indicative of acute Cd toxicosis or mortality in either group. The body mass gain of group Cd-75 chickens markedly decreased. Prolonged Cd exposure of the cockerels increased the relative mass of the liver and heart and markedly decreased that of the testes. The change in mass was proportional to the Cd load. The Cd-fed chickens developed focal pathological fatty infiltration of the liver, histiocytic infiltration of the jejunal mucosa and focal lympho-histiocytic interstitial infiltration and fibrosis of the kidney, which supports the view that prolonged Cd exposure leads to the development of subacute-chronic tissue changes in the kidney. The Cd content of the organs increased by one to three orders of magnitude, in direct proportion to the Cd load. The Cd content of most organs was 2 to 3 times as high as the value reported for broilers exposed to a similar Cd load lasting for 68 days (Bokori et al., 1995*b*). This indicates that the degree of Cd accumulation is markedly influenced by the duration of the Cd exposure. The highest Cd content was demonstrated in the kidney (724 mg/kg of dry matter). The Cd exposure markedly lowered the Zn, Mo and B content of the bones and the Ni content of the myocardium.

Key words: Broiler chickens, cadmium load, clinical signs, cadmium accumulation, gross lesions, histopathological changes

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The results of cadmium (Cd) loading trials of relatively short duration (37 and 68 days), in which Japanese quails and broiler chickens were fed a diet containing different levels of cadmium (Cd), have been reported in earlier papers (Bokori et al., 1995*a*; Bokori et al., 1995*b*). In addition to showing the adverse effects of Cd on egg production and body mass gain, the above-cited trials have revealed that the domestic fowl is only moderately susceptible to Cd, as indicated by the development of rather mild pathological lesions and histopathological changes in the organs of chickens fed a diet containing Cd at levels as high as 75–150 ppm for several weeks. For this reason and because, apart from our own experiment on two broiler chickens (Bokori et al., 1995*b*), there are very few reports in the literature on prolonged (several months long) Cd loading trials in poultry (Pritzl et al., 1974; Suelz et al., 1974; Leach et al., 1979; Sharma et al., 1979; Stolley, 1989), we conducted a trial in which a relatively large number of broiler chickens were fed a diet of moderately high Cd content (25 and 75 ppm, respectively) up to 41 weeks of age.

The objective of the experiment was to determine, after a moderately severe Cd exposure of more than 9 months duration, the concentrations of Cd in the organs accumulating that element, to describe the gross lesions and histopathological changes developing in the different organs, and to look for a correlation, if any, between the severity of the pathological and histopathological changes and the duration of Cd exposure.

Materials and methods

A total of 40 Ross meat-type hybrid cockerel chicks were used. The chicks were pre-reared for 2 weeks, then 26 cockerel chicks of approximately identical body mass were selected and divided into three groups (n = 6, 10 and 10, respectively).

The birds were marked with metal wing numbers and housed in a chicken house of controlled microclimate, in meat-type chicken rearing batteries so as to eliminate any differences in temperature, relative humidity and lighting. The chickens were kept and fed and the feed analyses were performed in precisely the same manner as described earlier (Bokori et al., 1995*a*).

In the 14-day pre-rearing period the chickens received a semi-intensive poultry starter diet (Table 1). Subsequently they were fed, throughout the trial period, a poultry grower diet of the composition and content shown in Table 2. The diet fed to the control birds (Cd-0, n = 6)), which did not contain Cd in detectable quantities (0.03 mg/kg), was not supplemented with Cd. The diet of the two experimental groups (n = 10 each) was supplemented with 25 mg and 75 mg Cd per kg, respectively, which was homogeneously mixed in the feed in the form of crystalline $CdSO_4$ (Table 3).

Table 1

Composition and laboratory assay of the broiler starter diet

Composition		kg
Maize		43.00
Wheat		22.00
Extracted soybean	(48%)	25.00
Fish meal	(65%)	3.00
Energomix-40 (40% fat)		4.00
Limestone		1.30
AP-17 (Ca–P supplement)		0.90
Salt		0.30
Standard poultry grower premix		0.50
Total		100.00
Laboratory results		
Dry matter	%	94.60
Crude protein	%	21.80
Crude fat	%	5.07
Crude fibre	%	3.30
Crude ash	%	6.10
Ca	%	1.51
P	%	0.94
Na	%	0.19

Table 2

Composition of the broiler grower diet

Composition		kg
Maize		40.00
Wheat		32.00
Extracted soybean	(48%)	20.00
Fish meal	(70%)	3.50
Energomix-40 (40% fat)		1.50
Limestone		1.30
AP-17 (Ca–P supplement)		0.90
Salt		0.30
Standard poultry grower premix		0.50
Total		100.00

Table 2 continued

Some parameters of the control and the Cd-containing grower diets

Diet	Dry matter %	Crude protein %	Crude fat %	Crude fibre %	Crude ash %
Cd-0 (control)	90.10	18.90	2.18	2.80	5.20
Cd-25	89.70	18.60	2.40	2.95	5.37
Cd-75	90.20	18.50	2.75	3.05	5.70

Table 3

Experimental design

Group	No. of chickens	Treatment (mg Cd/kg of feed)
Cd-0 (control)	6	0
Cd-25	10	25
Cd-75	10	75

Feed and drinking water were available to the chickens *ad libitum*. The feeding trial was started when the birds were 14 days old and it was continued up to the end of the 41st week of life; thus, the duration of treatment was 274 days.

The clinical status of the birds was checked regularly throughout the experiment. The birds were weighed weekly up to week 17 and then monthly up to the end of the trial. The body mass data of the birds were added up in each group (Table 4). Up to 7 weeks of age the feed conversion efficiency of the chickens was also determined. The chickens that died or were culled during the trial were subjected to detailed gross pathological and in some cases also to microbiological examination. At the end of the feeding trial, when the chickens were 41 weeks old, all the remaining chickens were exsanguinated in anaesthesia, necropsied, and five of their organs (heart, liver, testis, spleen and brain) were weighed separately and their relative mass was calculated. Subsequently, samples were taken from 9 organs (myocardium, liver, lungs, kidney, testis, spleen, skeletal muscles, femur and brain) for the determination of 21 elements (Ca, P, Mg, S, Na, K, Fe, Cu, Zn, Mn, Mo, Cd, Ni, Co, Se, Al, Sr, Ba, B, Cr, Pb), as well as from 11 organs (liver, kidney, spleen, lungs, heart, brain, testis, skeletal muscle, jejunum, caecum, sternum) for light microscopic examination.

The mineral element content of organs was determined as described in an earlier paper (Bokori et al., 1993). The mineral element concentrations of the different organs, expressed as mg/kg of dry mass, were summarized in tables.

Table 4

Body mass of the control and Cd-fed groups of chickens (in g) between 2 and 41 weeks of age

Groups		Week 2	Week 4	Week 6	Week 8	Week 10	Week 12	Week 14
Cd-0	n	6	6	6	6	6	4	4
(control)	Mean	301.7	906.7	1,738.3	2,655.0	2,896.0	3,395.0	3,570.0
	SD	52.7	58.2	100.0	188.0	491.1	616.8	1,104.2
	CV	17.5	6.4	5.8	7.1	16.9	16.8	30.9
	SEM	21.5	23.8	41.9	76.8	200.5	308.4	552.2
Cd-25	n	10	10	10	10	9	8	8
	Mean	285.0	828.0	1,589.0	2,390.0	2,925.0	3,381.2	4,236.2
	SD	110.7	218.8	263.1	420.3	1,325.8	249.0	244.7
	CV	38.9	26.4	16.6	17.6	45.3	7.4	5.8
	SEM	35.0	69.1	83.2	132.9	419.2	88.0	86.5
Cd-75	n	10	10	10	10	9	9	8
	Mean	305.0	667.0	1,159.0	1,376.0	1,760.0	1,978.9	2,203.7
	SD	106.7	182.1	365.4	389.8	468.8	365.7	475.9
	CV	34.9	27.3	31.5	28.3	26.6	18.5	21.6
	SEM	33.7	57.6	115.5	123.3	156.3	121.9	168.3

Groups		Week 16	Week 17	Week 20	Week 24	Week 28	Week 36	Week 41
Cd-0	n	3	2	2	2	2	1	1
(control)	Mean	4,130.0	5,115.0	5,315.0	5,270.0	5,270.0	6,160.0	6,100.0
	SD	266.2	625.0	545.0	535.0	610.0	–	–
	CV	6.5	12.2	10.6	10.0	11.6	–	–
	SEM	153.7	441.9	385.4	378.3	431.3	–	–
Cd-25	n	8	8	5	5	5	5	5
	Mean	4,916.2	5,222.5	5,300.0	5,378.0	5,346.0	5,496.0	5,580.0
	SD	356.9	486.7	443.9	368.8	297.4	202.9	203.9
	CV	7.7	9.3	8.9	6.9	5.6	3.7	3.7
	SEM	126.2	172.1	198.5	164.9	133.0	90.8	91.2
Cd-75	n	8	8	6	5	4	3	3
	Mean	2,878.7	3,162.0	3,760.0	4,040.0	4,317.5	5,173.3	5,400.0
	SD	558.0	769.1	432.8	521.8	550.9	371.9	355.9
	CV	19.4	24.3	11.5	12.9	12.8	7.2	6.6
	SEM	197.3	271.9	176.7	233.9	275.5	214.7	205.9

For light microscopy, organ samples of suitable size were fixed in buffered formaldehyde solution, then frozen and paraffin-embedded sections were made from them and examined. The sections were stained with haematoxylin and eosin for general information and by Fat Red staining for assessment of the lipid content.

Results

The body mass data of the three groups of chickens between weeks 2 and 41 are summarized in Table 4 and in Figs 1–4. The weekly feed utilization of the chickens, as calculated from the feed consumption measured at the age of 3–7 weeks, is shown in Fig. 5. Tables 5 and 5A present the average concentrations (mg/kg of dry matter) of 17 mineral elements (macro- and microelements) measured in 9 different organs of the birds. The concentrations of additional four elements (Mg, Na, Co and Cr) were measured but are not presented, partly because their concentration did not show appreciable changes, partly because it was below the limit of detection (Co, Cr).

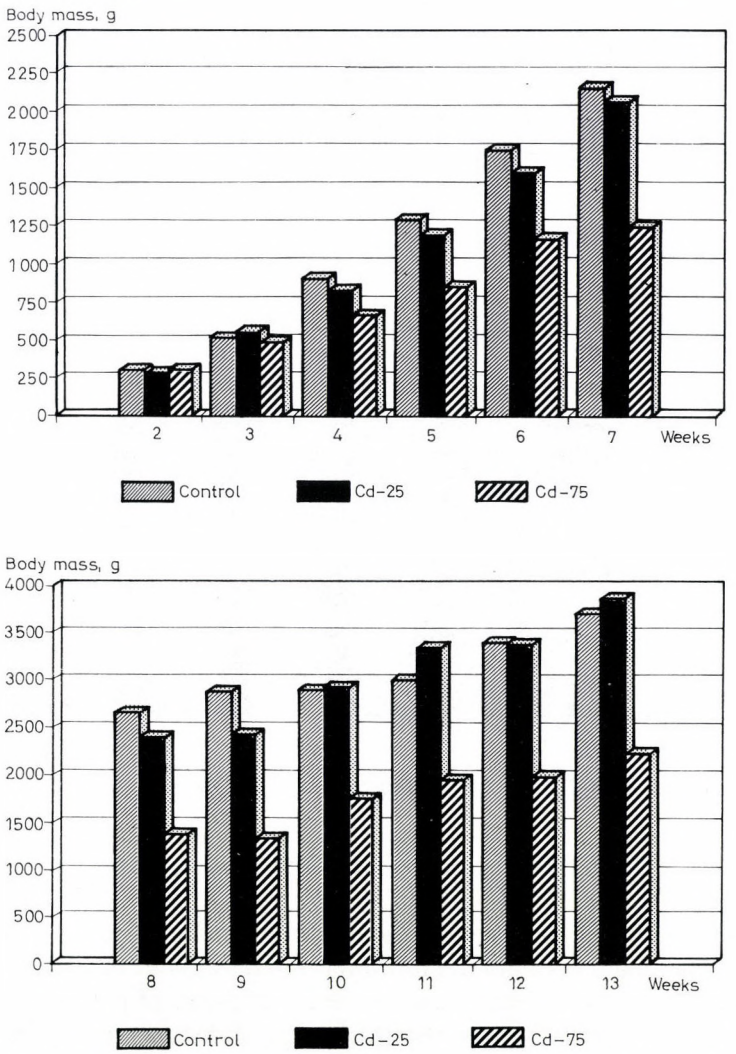
The pathomorphological lesions found in four different organs are shown in Table 6 and the relative mass of five organs (expressed in % of the body mass) in Table 7. The microbiological examinations were consistently negative. Some of the histopathological lesions found in the organs (kidney, jejunum and testis) of chickens are shown in Figs 6, 8 and 9 while the histological picture of the jejunum of a control bird in Fig. 7.

Discussion

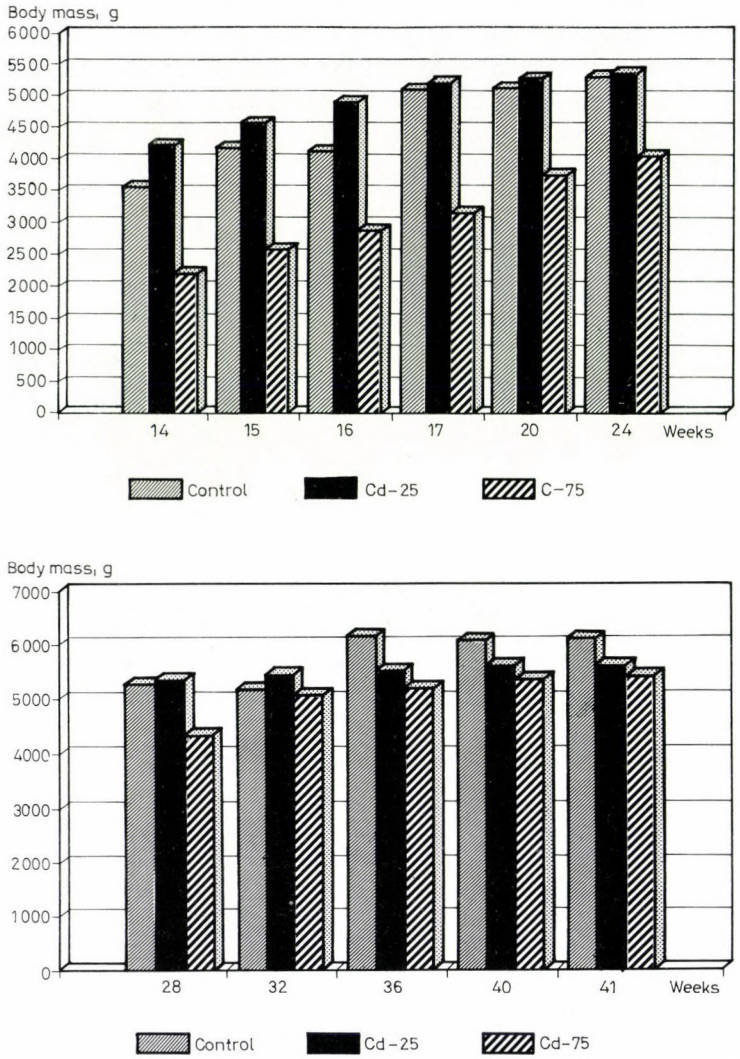
Up to the age of 10 weeks, the chickens of group Cd-0 (control) and Cd-25 did not exhibit any abnormal clinical signs, they grew well and showed good feathering. At week 10, their body mass reached 2,896 and 2,925 g, respectively. In the subsequent weeks 5 control birds died (one at week 11, 12, 15, 17 and 36 each) after having shown signs of locomotor disturbances, diarrhoea, lack of appetite and weakness for one or two days. Necropsy revealed enteritis, mild hepatic degeneration and pulmonary oedema in three out of the five dead birds. Bacteriological examination of the bone marrow was performed and yielded negative result in two of these chickens. Two dead chickens were unsuitable for gross pathological examination because of autolysis.

In group Cd-25, only one death occurred up to 10 weeks of age, when the average body mass of these birds exceeded that of the controls by 29 g. From weeks 3–4 of the trial, chickens of group Cd-75 showed slightly impaired appetite, their comb was pale, their feathers ruffled, and the birds were retarded in growth. At the end of the 4th week of life their average body mass was 667 g, compared to the 906.7 g average body mass measured in the control group. From that time up

to weeks 24–28, the body mass of Cd-75 chickens was markedly lower than that of the control chickens and those of group Cd-25, and between weeks 10 and 24 their number gradually decreased to half the initial group size. By weeks 40–41 the body mass of the surviving chickens increased again, and e.g. at week 41 their average body mass (5,400 g) was only 700 g less than that of the controls (6,100 g) (Table 4).



Figs 1–2. Body mass data of the control and the cadmium-fed (Cd-25, Cd-75) broiler chickens between weeks 2 and 13 of the trial



Figs 3–4. Body mass data of the control and the cadmium-fed (Cd-25, Cd-75) broiler chickens between weeks 14 and 41 of the trial

The data of weighings support our earlier observation (Bokori et al., 1995*b*) that the feeding of a diet containing low levels (in this case 25 ppm) of Cd does not cause pathological clinical signs and, in the initial phase of growth, it may even stimulate the birds' growth. However, the feeding of diets containing higher levels (e.g. 75 ppm) of Cd caused visible growth disturbances, anaemia and sporadic deaths already in the first weeks of the trial, but it did not give rise to mass mortality within a short time.

A realistic evaluation of the chickens' feed conversion efficiency was possible only up to 7 weeks of age. Namely, after that time the measurement of "specific" feed utilization would have been unrealistic because of the known physiological decrease in the feed conversion efficiency of poultry and also because of the decrease in the number of chickens as a result of mortality. In the first 7 weeks of life (except in week 4) the feed utilization of Cd-25 chickens was somewhat higher than that of the control birds. At that time the feed utilization was around 2.5 kg, which could be considered still acceptable under the given conditions. The feed conversion efficiency was much (by approx. 1 kg) lower in group Cd-75 (Fig. 5).

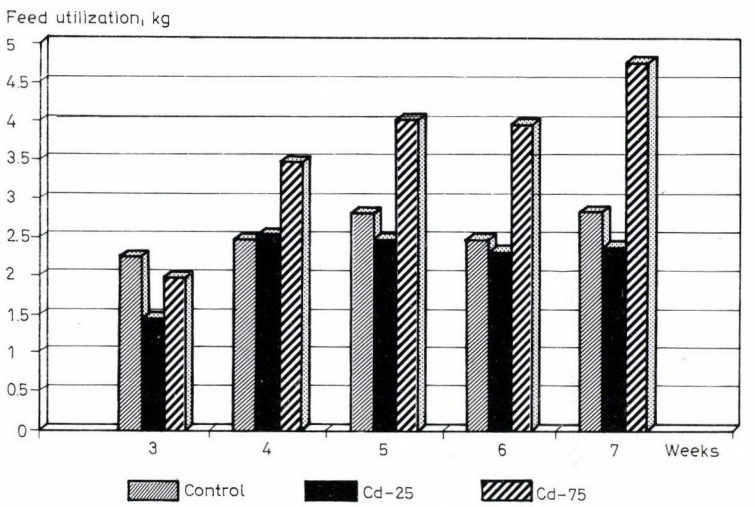


Fig. 5. Weekly feed utilization of the control and the cadmium-fed (Cd-25, Cd-75) broiler chickens between weeks 3 and 7 of life

Gross pathological examination of the 5 chickens that died in group Cd-25 between weeks 10 and 20 revealed anaemia, pulmonary oedema and, in one bird each, fibrinous pericarditis / peritonitis and perosis. In group Cd-75, 7 chickens died between weeks 10 and 29. Three of them had renal degeneration of different severity, two had pulmonary oedema and myocardial degeneration, and one showed ascites and hepatic degeneration. One dead bird was unsuitable for necropsy because of autolysis.

Gross pathological examination of one control and eight experimental chickens — all of which were well developed and in good body condition — exsanguinated at the end of the feeding trial revealed no pathological lesions in the control bird and greyish-white spots the size of a pinhead or hempseed in the slightly swollen, pale brown kidneys (nephrosis?) of most chickens of the two ex-

Table 5

Average concentrations of P, Ca, S, Cd, Fe, Cu, Zn, Mn, Mo and Se in nine different organs of control broiler chickens and those fed different levels of cadmium

Group	Organ	Element (mg/kg of dry matter)									
		P	Ca	S	Cd	Fe	Cu	Zn	Mn	Mo	Se
Cd-0	Myocardium	7,417	213	8,338	0,058	215.0	12.5	109.0	1.01	0.00	3.92
(control)	Liver	11,680	239	7,860	5,820	1,414.0	20.3	167.0	8.98	2.80	1.70
(n = 1)	Lungs	7,600	301	6,150	0,101	934.0	1.7	40.3	0.70	0.00	0.00
	Kidney	12,463	629	6,646	16,700	514.0	10.5	117.0	7.08	2.58	2.43
	Testis	15,417	739	5,101	0,822	87.9	6.1	74.0	1.70	0.00	4.89
	Spleen	14,450	569	7,861	0,032	1,029.0	3.1	74.2	1.21	0.00	2.60
	Skeletal muscle	8,270	175	7,151	0,107	54.2	2.6	62.4	0.64	0.00	2.60
	Bone	85,620	167,300	1,758	0,043	90.7	0.4	128.0	1.31	0.21	0.92
	Brain	10,940	593	4,452	0,413	72.5	9.7	148.0	1.41	0.00	3.30
Cd-25	Myocardium	7,470	168	7,986	2,112	190.2	11.7	104.6	0.99	0.00	1.68
(n = 5)	Liver	10,640	268	7,840	222,800	680.8	16.1	196.6	6.55	2.21	1.49
	Lungs	7,074	412	5,819	4,766	816.0	1.6	36.1	0.61	0.00	0.11
	Kidney	11,622	591	6,329	659,800	385.2	16.9	105.2	6.66	2.36	5.41
	Testis	16,620	744	5,498	7,856	65.3	6.1	89.7	1.47	0.00	2.99
	Spleen	14,166	261	7,442	8,504	808.8	3.0	76.3	1.06	0.00	4.57
	Skeletal muscle	7,436	250	7,338	2,558	59.7	2.9	66.0	0.84	0.00	3.54
	Bone	63,310	121,420	2,735	1,116	141.2	0.6	84.2	0.87	0.07	2.06
	Brain	11,606	1,293	4,618	0,487	80.4	9.5	85.7	1.22	0.00	4.45
Cd-75	Myocardium	7,254	221	7,666	4,977	232.7	10.4	105.3	0.95	0.00	1.73
(n = 3)	Liver	10,647	303	8,373	579,000	293.0	20.5	198.3	5.95	2.37	3.07
	Lungs	9,541	470	6,658	13,540	748.3	2.3	50.1	0.82	0.00	2.28
	Kidney	11,837	690	7,868	724,333	164.3	53.4	106.8	6.17	2.30	3.09
	Testis	15,063	725	5,676	25,367	60.5	5.3	96.2	1.47	0.00	2.46
	Spleen	12,397	603	6,647	20,067	499.0	3.1	66.9	1.19	0.00	1.43
	Skeletal muscle	7,742	210	7,518	4,560	20.9	1.5	42.4	0.45	0.00	2.63
	Bone	72,230	141,533	2,370	2,423	124.0	0.5	69.1	1.25	0.02	0.44
	Brain	9,959	2,727	3,629	1,642	62.9	10.3	147.5	1.38	0.00	2.72

Table 5A

Average concentrations of K, Ba, B, Pb, Ni, Sr and Al in nine different organs of control broiler chickens and fed with different levels of cadmium

Group	Organ	Element (mg/kg of dry matter)						
		K	Ba	B	Pb	Ni	Sr	Al
Cd-0	Myocardium	11,190	0.00	0.00	0.00	1.20	0.16	2.25
(control)	Liver	10,550	2.64	0.00	0.43	0.12	0.13	3.08
(n = 1)	Lungs	10,740	0.00	0.00	0.00	0.00	0.17	1.47
	Kidney	11,740	0.00	0.00	0.00	0.00	0.40	0.79
	Testis	18,730	0.00	0.00	0.00	0.00	0.05	4.07
	Spleen	16,580	0.00	0.00	1.14	0.00	0.12	5.26
	Skeletal muscle	11,830	0.00	0.00	0.00	0.34	0.42	2.62
	Bone	1,897	4.22	0.00	0.00	0.00	28.40	4.73
	Brain	12,130	0.00	90.60	0.09	16.30	1.64	10.60
Cd-25	Myocardium	10,978	0.00	0.00	0.09	0.16	0.03	1.34
(n = 5)	Liver	9,470	0.03	0.00	0.22	0.00	0.13	1.76
	Lungs	9,553	0.00	0.00	0.19	0.00	0.19	1.93
	Kidney	10,791	0.00	0.00	0.55	0.00	0.59	1.47
	Testis	19,864	0.01	0.00	0.42	0.49	0.42	2.48
	Spleen	15,390	0.00	0.00	0.22	0.08	0.51	3.92
	Skeletal muscle	11,846	0.00	0.00	0.47	0.17	0.60	3.44
	Bone	2,531	4.81	0.00	1.17	0.09	23.76	1.79
	Brain	12,990	0.00	10.50	0.25	7.76	1.59	6.91
Cd-75	Myocardium	10,523	0.00	0.09	0.00	0.00	0.14	1.91
(n = 3)	Liver	10,114	0.00	0.00	0.02	0.00	0.18	2.08
	Lungs	11,690	0.88	0.00	0.14	0.00	0.37	1.55
	Kidney	12,783	0.00	0.00	0.11	0.00	0.64	0.57
	Testis	19,323	0.13	0.15	0.30	0.00	0.18	2.10
	Spleen	14,073	0.00	0.01	0.14	0.00	0.16	4.47
	Skeletal muscle	12,880	0.00	0.05	0.00	0.02	0.60	7.29
	Bone	2,642	12.70	0.00	0.21	0.20	38.93	7.64
	Brain	10,580	0.40	12.78	0.60	19.06	2.51	10.28

Table 6

Pathomorphological lesions found in the control and experimental animals

Groups and chickens		Cd-0 (control)	Cd-25					Cd-75		
		301	270	295	719	725	736	271	313	356
Organs, lesions										
Liver:	pathological fatty infiltration			+	+	+		+	++	+
Kidney:	focal lympho-histiocytic interstitial inflammation	+		++	++	++	+	+++	++	+++
	fibrosis							+	++	++
Jejunum:	proliferative mononuclear cell infiltration of the mucosa		+			+		+++	+	
Testis:	decreased spermatogenesis, atrophy of the germinal epithelium							+++		
Tumour originating from the kidney: adenoma								+++		

+ = mild, circumscribed change; ++ = moderate changes seen in several places; +++ = severe, diffuse lesion

perimental groups. Primarily in group Cd-75 chickens, the testes were decreased in size and slightly flaccid to the touch. In the abdominal cavity of one chicken of group Cd-75, a spherical tumour-like mass approx. 8 cm in diameter, covered by serosa and starting from the kidney, was seen.

The average mass of the liver and heart was 1.62 and 0.53 g in group Cd-75, 1.25 and 0.48 in group Cd-25, and 1.02 and 0.38 g in the control group, respectively (Table 7), indicating that the relative mass of the liver and heart increased in direct proportion to the Cd load. The relative average mass of the testes showed a conspicuous decrease proportional to the Cd load (atrophy): it was 0.22 and 0.20 in group Cd-75, 0.27 and 0.27 g in group Cd-25, and 0.35 and 0.29 g in group Cd-0.

Table 7

Relative mass of organs of the control and experimental chickens (expressed as % of body mass)

Group	Organs						
	Chicken	Heart	Liver	Testes		Spleen	Brain
Cd-0 (control)	301	0.38	1.02	0.35	0.29	0.10	0.06
Cd-25	270	0.56	1.62	0.20	0.27	0.12	0.07
	295	0.49	1.21	0.30	0.24	0.11	0.06
	719	0.44	1.15	0.26	0.20	0.11	0.07
	725	0.48	1.20	0.32	0.30	0.15	0.06
	736	0.45	1.09	0.26	0.28	0.10	0.07
Average:		0.48	1.25	0.27	0.27	0.12	0.07
Cd-75	271	0.55	2.16	0.04	0.03	0.14	0.08
	313	0.43	1.26	0.30	0.28	0.07	0.07
	356	0.62	1.44	0.32	0.30	0.13	0.06
Average:		0.53	1.62	0.22	0.20	0.11	0.07

Light microscopic examination revealed simple pathological fatty infiltration of the hepatocytes in circumscribed areas of the liver in most chickens of groups Cd-25 and Cd-75 (Table 6). In the kidneys of all chickens but one, there were changes indicative of focal lympho-histiocytic interstitial nephritis. These changes were more severe and extended to larger areas in the kidneys of chickens of group Cd-75, in which fibrosis was also present (Fig. 6). Half of the chickens of the Cd-treated groups showed an infiltration of the lamina propria of the jejunal

mucosa by large numbers of histiocytes. As a result, in the affected areas the intestinal villi were larger than in the controls (Fig. 7), and their tissue structure was less distinct (Fig. 8). In the testes of one chicken of group Cd-75 the total absence of spermatogenesis and changes indicative of atrophy of the germinal epithelial cells were observed (Fig. 9). The subacute-chronic histopathological changes found in the organs are similar to those seen by us earlier in the organs of two cockerels exposed to a prolonged Cd load (Bokori et al., 1995*b*). The tumour-like mass found in the abdominal cavity of one of the chickens in group Cd-75 histologically proved to be an adenoma starting from the kidney.

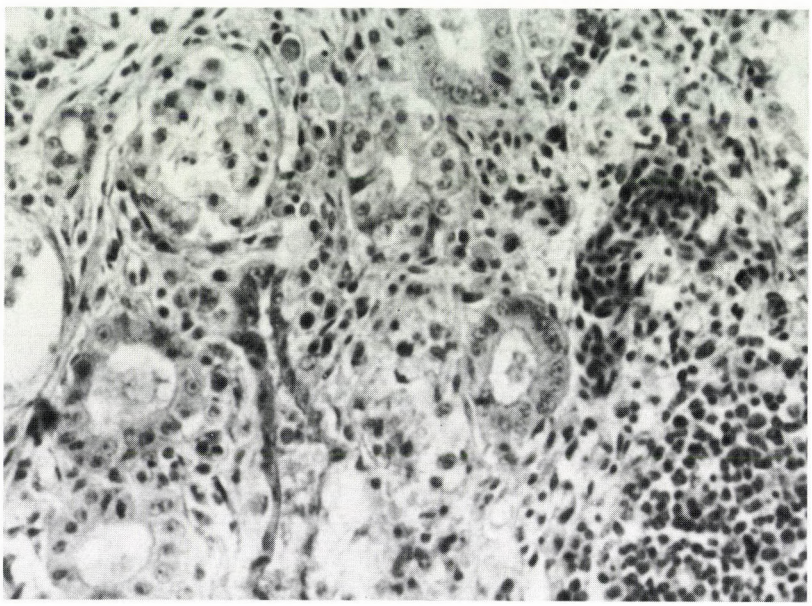


Fig. 6. Subacute interstitial nephritis with fibrosis and atrophy of the parenchyma. Haematoxylin and eosin (H.-E.), × 200

The Cd content of organs assayed in order to confirm the accumulation of minerals increased in direct proportion to the load, usually by one (kidney, skeletal muscles, brain) or two orders of magnitude (myocardium, liver, lungs) (Table 5). The biggest, more than 620-fold, concentration increase of Cd was found in the spleen. The highest Cd concentration was obtained in the kidney (724 mg/kg of dry matter) and in the liver (579 mg/kg of dry matter).

These results are consistent with the findings of Sharma et al. (1979) who also measured the highest Cd concentrations in the kidney and liver after a prolonged (6-month) uptake of 13.1 ppm Cd. Similar results were obtained by Überschär et al. (1982) in 6-week Cd loading trials in broiler chickens.

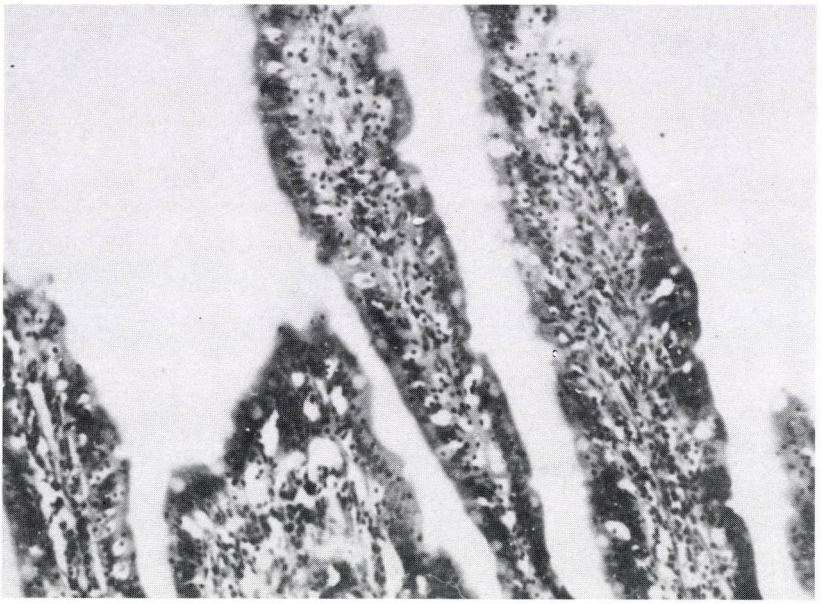


Fig. 7. Segment of jejunum from a control cockerel. The intestinal villi are intact. H.-E., × 63

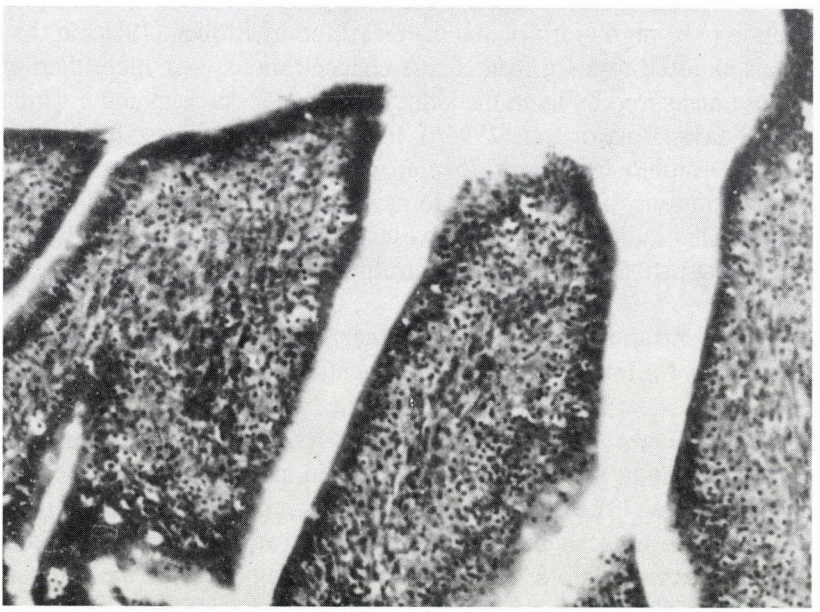


Fig. 8. Segment of jejunum from a cockerel fed a diet containing 75 ppm Cd. Note enlargement of the intestinal villi because of histiocytic infiltration. H.-E., × 63

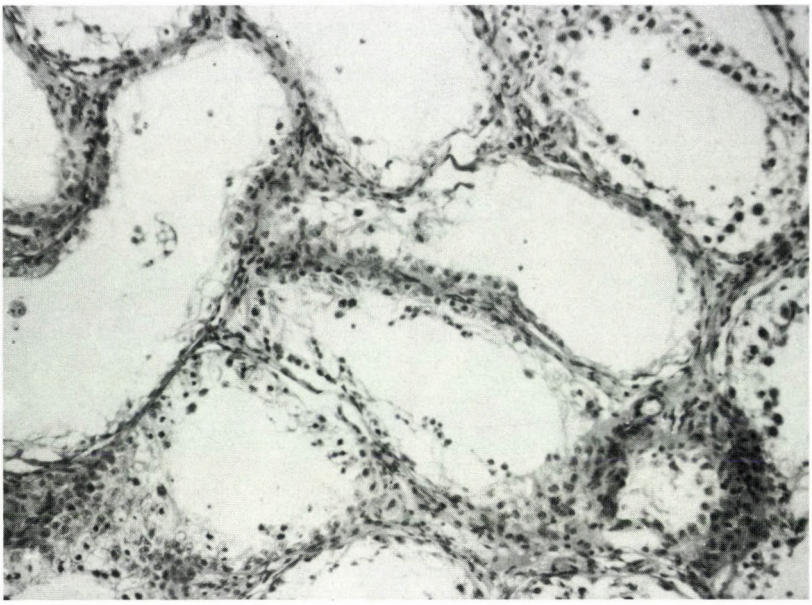


Fig. 9. Testis from a cockerel fed a diet containing 75 ppm Cd. Note atrophy of the germinal epithelium and the absence of spermatogenesis. H.-E., × 200

The Cd concentrations found in the kidney are consistent with the lowest Cd concentration (616 mg/kg of dry matter) measured by Stolley (1989) in the kidney of cockerels exposed to a Cd load. These concentrations were more than twice as high as those measured by us in the kidneys of broiler chickens fed a similar dose of Cd for 68 days (Bokori et al., 1995*b*). In general, it can be established that after a more than 9-month feeding of 75 ppm Cd all organs contained 2 to 3 times higher concentrations of Cd than that found in the same organs of chickens fed Cd for 68 days. This indicates that, as was to be expected, the accumulation of Cd increases in proportion to the Cd concentration of the diet and also to the duration of Cd exposure.

The concentration of some other elements in certain organs also changed in proportion to the Cd load. Of the macroelements, the P concentration of the bones markedly decreased while the Ca content of the brain considerably increased. The S content of the bones also tended to increase. As regards the trace elements, the substantial decrease in the B content of the brain and the Ni content of the myocardium was striking (Table 5A). The concentration of Fe decreased in the kidney, spleen and skeletal muscles but primarily in the liver, whereas the Cu content of the kidneys increased fivefold. The Zn content of the bones and the Mn content of the liver dropped almost to half. A substantial (approx. tenfold) decrease occurred

in the Mo content of the bones as well. The Se content of the myocardium also decreased appreciably.

Summing up the clinical observations and the results of gross pathological and histopathological examinations, it can be established that a diet containing 25 or 75 ppm Cd, when fed for more than 9 months, did not cause clinical signs indicative of acute toxicosis. While chickens fed 25 ppm Cd transiently showed a body mass gain somewhat higher than the controls, the body mass gain of chickens fed 75 ppm Cd was definitely impaired, especially in the first two thirds of the trial. This, however, was not accompanied by the appearance of typical clinical signs indicative of Cd toxicosis. These findings are partially consistent with the observations of Leach et al. (1979) who reported that 48 ppm Cd, when fed to 6- to 7-month-old laying hens for 48 weeks, was only slightly toxic.

The exposure of cockerels to Cd administered via the feed caused an increase in the relative mass of the liver and heart and a substantial decrease in the relative mass of the testes. These changes were proportional to the dose of Cd fed. In the Cd-fed chickens a focal pathological fatty infiltration of the liver and histiocytic infiltration of the jejunal mucosa developed. In addition, focal lympho-histiocytic infiltrations appeared in the kidney in large number and extending to large areas, and these lesions were in some cases accompanied by focal fibrosis. All this indicates the dominance of subacute or chronic lesions during prolonged Cd load, primarily in the kidney. The Cd content of the different organs increased in direct proportion to the dose applied, usually by one or two orders of magnitude. The biggest (more than 620-fold) increase in Cd concentration occurred in the spleen, while the highest Cd concentration (724 mg/kg of dry matter) was detected in the kidney. The Cd concentration of the organs assayed was usually 2 or 3 times higher than the levels measured by us earlier in chickens exposed to a similar Cd load but for a shorter (68-day) period. This observation underscores that the duration of exposure markedly influences the degree of Cd accumulation. The feeding of Cd markedly decreased the Zn, Mo and B content of the bones and the Ni content of the myocardium.

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HAEMATOLOGIC VALUES OF HYLA RABBITS FED DIFFERENT LEVELS OF BY-PRODUCT ARISING FROM THE PROPAGATION OF *PLEUROTUS PULMONARIUS*

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The nutritive influence of different levels of by-product arising from the propagation of the mushroom *Pleurotus pulmonarius* on the red and white blood cell count of rabbits was studied in the course of fattening. The experiment was carried out on 4 groups of male Hyla rabbits, a control group (C) and 3 experimental groups (E_1, E_2, E_3), with 15 rabbits per group. The control group was fed a standard fodder and the three experimental groups were fed the standard mix with 10%, 20% or 30% dried by-product added, respectively. At the end of the 9th week of the experiment, blood samples were obtained by cardiac puncture, and the red blood cell (RBC) count, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), haemoglobin, haematocrit, thrombocyte count, white blood cell (WBC) count and the differential WBC were determined. The different levels of by-product interwoven by mycelium added to the feed did not change the RBC count but significantly increased the MCV, MCH, haemoglobin concentration and the haematocrit value. The different diets had no influence on either the WBC count or the differential WBC count, but the number of thrombocytes decreased significantly.

Key words: Hyla rabbits, feeding, by-product of mushroom growing, *Pleurotus pulmonarius,* haematologic parameters, red blood cell count, white blood cell count

Agricultural production of corn, barley, wheat and oats yields not only grain but also large quantities of secondary products such as corn stalks, straw, etc. These products can be used in the nutrition of cattle, especially as feed for ruminants and other herbivores. It is a well-known fact that these secondary products are poorly utilised in the digestive system of other species of animals. The basic limiting factors are the presence of large quantities of lignin, cellulose, hemicellulose, aromatic matters, etc. (Gioffre et al., 1988). In order to increase the digestibility of such potential feeds for rabbits (Lebas et al., 1978; McDonald et al., 1978; De Blas et al., 1979; Auxilia, 1981; Jensen et al., 1986; Radwan et al., 1987; Gioffre et al., 1988; Mužic et al., 1990) and ruminants (Hartley, 1987;

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Henics, 1987; Zadražil, 1987), researchers have treated them with NaOH, NH_3, CO_2, SO_2, etc., but with no particular success and without achieving a broad application in the practice. A biological approach to increasing the digestibility of straw and other secondary agricultural products could be the decomposition of the plant cell wall by means of the mycelium of *Pleurotus* sp. and other fungus species. Experiments have been carried out (Rai and Gupta, 1989; Mužic et al., 1990; Božac et al., 1991; Rupić et al., 1991) in which the by-product of mushroom growing (interwoven, exhausted substrate) was used as a feed component for rabbits. It was established that wheat straw on which *Pleurotus* fungi had been grown became more digestible (Zadražil, 1987; Mužic et al., 1990) than untreated straw. In our earlier investigations (Mužic et al., 1990; Božac et al., 1991) we could not find any negative influence of incorporating different quantities of substrate interwoven by mycelium into the feed on the growth, feed consumption and feed conversion of Chinchilla and Hyla rabbits after 63 days of experimental fattening.

The aim of the present study was to determine the influence of different levels of the dried fungal by-product in the feed on the red and white blood cell count and the health status of Hyla rabbits after 63 days of experimental feeding.

Materials and methods

Sixty male, weaned Hyla rabbits (age: 30 days) were used. The rabbits were randomly divided into 4 groups of 15 animals each: a control group (C) and three experimental groups (E_{1-3}). The initial average body weight of rabbits in the control group (C) was 616 g, while in the three experimental groups it was 626 g (E_1), 617 g (E_2), and 632 g (E_3). Analysis of variance showed that differences in the initial body weight of rabbits assigned to the different groups were not significant ($P > 0.05$). Each group of 15 rabbits was housed in one of four stainless steel cages, located in the same room and on the top of each battery. The room was air-conditioned, thus the temperature was maintained between 18 and 20 °C, and the relative humidity between 60 and 70%. To decrease the risk of infection, all cages and equipment were treated with a chlorine disinfectant (Izosan® G) before the experiment.

The pelleted by-product was obtained from the commercial production of *Pleurotus pulmonarius*. By means of tissue culture, the Oyster mushroom culture spawn was developed, propagated on wheat grain and sown in the moistened and pasteurised wheat straw. The inoculated substrate (straw+spawn) was packed in polyethylene bags and interwoven for 20 days at 22 °C. After picking the mushroom (yield: 20%) the substrate residue (straw+spawn) was dried and ground in a hammer mill. The floury material was mixed with the other components into fod-

der mixes which were then pelleted with no binder added. The chemical composition and metabolic energy content of the fodder mixes are shown in Table 1.

The rabbits in the control group (C) were not fed the fungal by-product. The level of fungal residue in the diet of the experimental groups was 10% (E_1), 20% (E_2) and 30% (E_3). The dried by-product arising from the propagation of *Pleurotus pulmonarius* contained 11.38% water, 6.79% crude protein, 1.33% crude fat, 25.52% crude fibre, 48.42% nitrogen free extract, and 4.56% ash.

Table 1

Metabolizable energy content and basic chemical composition of fodder mixes (%)

Chemical composition	Groups			
	C	E_1	E_2	E_3
Moisture	10.10	10.39	10.53	10.61
Crude protein	17.25	17.24	17.09	17.35
Crude fat	6.95	6.79	7.52	8.52
Crude fibre	14.11	14.09	14.05	14.33
Ash	5.98	5.74	5.37	5.11
Nitrogen free extract	28.39	31.28	33.73	35.44
Ca	0.63	0.72	0.70	0.71
P	0.54	0.54	0.45	0.41
Metabolizable energy, MJ/kg	10,098	10,103	10,090	10,111

Microbiological examination of one gram of fodder mix showed a germ count of 1,200,000 for the control group (C), 1,120,000 for E_1, 900,000 for E_2, and 1,300,000 for E_3. In the same quantity (1 g) of fodder mix no salmonellae were found, while the anaerobic count was 1,000 and the total mould (*Ascomycetes, Phycomycetes*) count was also 1,000 in all groups.

Feed and drinking water were provided *ad libitum* for 63 days. At the end of the experiment, blood samples for haematological analyses were obtained by cardiac puncture. The blood was stored in Greiner test tubes with EDTA. The red blood cell (RBC) count, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), haemoglobin concentration, haematocrit value, thrombocyte count, white blood cell (WBC) count and differential WBC count were determined 1 h after taking the blood samples. Blood samples were taken from all rabbits in the experiment, each sample in a separate test tube, and then subjected to the above-mentioned determination of haematologic parameters. In some test tubes (12–15 blood samples) coagulation or haemolysis took place; such samples were excluded from

the experiment. All haematologic parameters were determined with the help of reagents and using the Technicon H-1 apparatus.

The mean values and the standard deviations were calculated (Snedecor and Cochran, 1967). The significance of the difference between the mean values of haematologic parameters was calculated by Student's *t*-test. The animals used in this study were maintained in facilities approved by the Croatian Association for Accreditation of Laboratory Animal Care and in accordance with the current regulations and standards of the Croatian Department of Agriculture.

Results

After 63 days of feeding the following mean body weights were attained: 2044 g (C), 2038 g (E_1), 2133 g (E_2), and 2120 g (E_3). The haematologic values of Hyla rabbits (age: 93 days) after 63 days of feeding are shown in Table 2 and the differential white blood counts in Table 3. There were no statistically significant discrepancies either between the control (C) and the experimental groups (E_{1-3}) or among the experimental groups in the RBC count, MCHC, and the WBC count ($P > 0.05$).

The rabbits of all experimental groups (E_{1-3}) had a higher MCV than those of the control group. The MCV of the first (E_1) and third (E_3) group was significantly higher than that of the control group, and the MCV of the third group (E_3) was also significantly higher than that of the second experimental group (E_2, $P < 0.05$). The rabbits of the experimental groups (E_{1-3}) also had a higher MCH than those of the control group, and the MCH of the third group (E_3) was also significantly higher than that of the control and the second experimental group (E_2, $P < 0.05$).

The relation of MCH values among the groups was very similar to that of the MCV values. The biggest increase in MCV and MCH was found in the third experimental group (E_3) which received a feed of 30% dried by-product level. The rabbits of all experimental groups (E_{1-3}) had higher haemoglobin concentration than the controls, and the animals of group E_3, consuming a feed of 30% by-product content, had a significantly higher haemoglobin concentration than those of groups C, E_1 and E_2 ($P < 0.05$). The lowest haematocrit value was found in rabbits of group C while the highest in E_3 which had significantly higher haematocrit values than groups C, E_1 and E_2 ($P < 0.05$).

The control rabbits had a significantly higher thrombocyte count than those in the experimental groups ($P < 0.05$). The analysis of differential white blood cell count (Table 3) showed no significant differences in the percentage and number of neutrophils, basophils and eosinophils either between the control and the experimental groups (E_{1-3}) or among the experimental groups ($P > 0.05$). However,

Table 2

Haematologic values of Hyla rabbits at the end of the experiment (63 days)

Statistical data	Groups							
	C (n = 8)		E_1 (n = 13)		E_2 (n = 12)		E_3 (n = 15)	
	mean	SD	mean	SD	mean	SD	mean	SD
RBC, 10^{12}/L	5.79	0.48	5.59	0.46	5.70	0.30	5.74	0.32
MCV, f/L	63.56^{ab}	3.30	67.80^{a}	4.74	66.64^{c}	4.28	68.96^{bc}	1.96
MCH, pg	20.18^{a}	1.22	21.36	1.80	21.04^{b}	1.51	22.30^{ab}	0.85
MCHC, %	31.75	0.83	31.52	1.31	31.58	1.66	32.35	1.25
Haemoglobin, g/L	117.87^{a}	6.87	119.15^{b}	8.38	119.83^{c}	6.73	128.13^{abc}	7.62
Haemotocrit, L/L	0.370^{a}	0.01	0.378^{b}	0.01	0.378^{c}	0.18	0.396^{ab}	0.03
WBC, 10^{9}/L	8.57	1.58	6.75	1.41	7.59	3.07	6.11	1.50
Thrombocytes, 10^{9}/L	664.62^{abc}	239.59	471.38^{a}	117.89	401.75^{b}	135.59	394.86^{c}	130.20

Explanation: a:a, b:b, c:c = $P < 0.05$ (significance of difference in mean values of haematologic parameters between groups of rabbits)

Table 3

Differential white blood count of Hyla rabbits at the end of the experiment (63 days)

Statistical data		Groups							
		C (n = 8)		E_1 (n = 11)		E_2 (n = 12)		E_3 (n = 14)	
		mean	SD	mean	SD	mean	SD	mean	SD
Neutrophils,	%	28.80	18.71	33.58	13.35	24.35	18.41	24.51	15.15
	10^9/l	2.45	1.45	2.19	0.97	1.59	1.12	1.47	0.93
Basophils,	%	2.58	1.01	2.92	0.84	2.76	1.61	3.81	3.36
	10^9/l	0.22	0.09	0.18	0.06	0.21	0.14	0.25	0.30
Eosinophils,	%	0.56	0.19	0.81	0.72	0.96	0.70	0.67	0.27
	10^9/l	0.04	0.02	0.05	0.04	0.06	0.05	0.04	0.02
Lymphocytes,	%	50.65	10.55	52.08	9.94	44.33[a]	9.14	53.19[a]	11.51
	10^9/l	3.69	1.13	3.46	0.96	3.39	1.60	3.29	0.88
Monocytes,	%	21.22	22.87	11.41[b]	7.61	26.15[b]	18.71	18.39	15.74
	10^9/l	2.29	5.97	0.80[c]	0.61	2.14[c]	2.06	1.24	1.24

Explanation: a:a, b:b, c:c = $P < 0.05$ (significance of difference in mean values of different categories of white blood cells between groups of rabbits)

rabbits in group E_3 had a significantly higher percentage of lymphocytes than those in group E_2, and rabbits of group E_2 had a significantly higher percentage and number of monocytes than those of group E_1 ($P < 0.05$).

Discussion

No significant differences were found in RBC count either between the control group and the experimental groups (E_{1-3}) or among the experimental groups of Hyla rabbits. At the first glance, this would suggest that dietary by-product levels of 10%, 20%, or 30% had no influence on erythropoiesis after 63 days of experimental feeding. However, the rabbits of all experimental groups fed the substrate had a greater MCV as compared to the controls. From the significant difference ($P < 0.05$) found in MCV values between the controls and groups E_1 and E_3 one can draw the conclusion that the feeding of substrate exerted an effect on erythropoiesis. The increase of MCV is reflected in the haematocrit value: the controls had the lowest value which gradually increased in the experimental groups, and was significantly higher ($P < 0.05$) in group E_3 than in the other groups (C, E_1, E_2). The MCV and the haematocrit values obtained demonstrate the influence exerted by the consumed mycelium on bone marrow activity.

This assertion is further corroborated by the haemoglobin levels found in the different groups, which were fully consistent with the MCV and haematocrit values. Haemoglobin concentration was the lowest in the control rabbits. In the experimental groups that value tended to increase from the first (E_1) to the third (E_3) group, where it was the highest. In other words, Hyla rabbits fed the highest level of fungal by-product had significantly higher haemoglobin concentration ($P < 0.05$).

An intensified activity of unipotent mother cells of the erythrocyte line that could lead to the above-mentioned values could be ascribed to elevated levels of erythropoietin, the erythropoiesis-regulating hormone. Erythropoietin is synthesized in cells of the kidney and liver under hypoxic conditions. According to Ganong (1991), the synthesis of erythropoietin is stimulated by the deoxy form of the haem protein: the protein in deoxy form enables erythropoietin gene transmission while that in oxy form hinders it. Hypoxia in rabbits could arise because of the reduced possibility for haemoglobin oxygenation, or due to a decreased affinity of haemoglobin to oxygen following the change in blood pH (Bohr effect).

Fungi, bacteria, and some plants contain the thermolabile enzyme thiaminase, which breaks down vitamin B_1. In animals that consume feeds containing the above-mentioned constituents, symptoms of vitamin B_1 deficiency develop (Duke, 1977; Scheunert and Trautmann, 1987). Pyruvic acid and its reduced form, lactic acid, are accumulated in the blood and tissues of such animals (McDonald et al., 1978; Church and Pond, 1988). The increase in the erythropoietic segment of bone

marrow obviously occurred to compensate for the slightly reduced oxygen-binding capacity of haemoglobin; therefore, the highest values of MCV, MCH, MCHC, haemoglobin and haematocrit were found in group E_3. These haematologic values of all groups (C, E_{1-3}) were similar to values obtained in earlier investigations (Hinton et al., 1982; Bortolotti et al., 1989; Rupić et al., 1991) and to the physiological values proposed for rabbits (Kolb, 1962; Spörri and Stünzi, 1969; Duke, 1977; Scheunert and Trautmann, 1987).

The number of thrombocytes in the blood of controls was significantly higher ($P < 0.05$) than in the experimental groups; within the latter, it decreased in proportion to the level of by-product in the diet, and thus the minimum value was found in group E_3. Based upon these results one could assume that the observed intensified activity of the bone erythrocyte line had a suppressive effect on the formation of megakaryocytes.

Similar trends were observed for the white blood cells, i.e. their maximum number was found in the controls (C) while the minimum number in group E_3. It may be supposed that the compensatory activity of the erythropoietic segment of bone marrow exerted a suppressive effect on cells of the myeloid line, in the same way as it did on the megakaryocytes. Histological studies would be needed to corroborate that assumption.

White blood cell and thrombocyte counts obtained in all groups of rabbits were in the physiological range and were very similar to the values reported for rabbits by other authors (Spörri and Stünzi, 1969; Duke, 1977; Hinton et al., 1982; Scheunert and Trautmann, 1987; Bortolotti et al., 1989).

The differential WBC obtained in two cases was difficult to explain. Namely, the highest percentage (but not the highest absolute number) of lymphocytes was found in group E_3 and the highest monocyte percentage and count in group E_2. As no aberrations had been expected in the differential blood count of rabbits used in the experiment, no additional (immunological, histopathological, pathological) analyses were carried out which could have explained the aberrations found in the differential blood count of rabbits fed different levels of fungal by-product. At any rate, consideration should to be given to that problem in future research aimed at investigating the influence of dietary fungal substrate on the health status of rabbits and other animals.

The differential white blood cell count of Hyla rabbits used in this study differed to some extent from that found in the experiments cited above. The percentage of neutrophils was considerably lower and that of monocytes considerably higher than the values reported by other authors (Spörri and Stünzi, 1969; Hinton et al., 1982; Scheunert and Trautmann, 1987; Kabata et al., 1991), while the percentages of basophils, eosinophils and lymphocytes were within the physiological limits reported for rabbits.

Daily clinical examination of all the rabbits revealed no alteration in the health status of either the control or the experimental animals. Based upon the

haematologic and daily clinical examinations of all rabbits used in the experiment, it can be concluded that the feeding of different (10%, 20%, 30%) levels of mycelium-interwoven substrate did not considerably affect the growth, the RBC and WBC counts or the general health status of Hyla rabbits fattened up to the age of 93 days.

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THE EFFECT OF ILOPROST ON PEROXIDATION IN DOG ISCHAEMIC MYOCARDIUM

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The aim of this study was to investigate the effect of Iloprost (Schering AG), a stable prostacyclin analogue, on the intensity of thiobarbituric acid-reactive substances (TBARS), conjugated diene and hydroperoxide production in ischaemic dog myocardium. Adult male mongrel dogs were divided into groups as follows: A = sham/operated group, B = control animals, and C = Iloprost-supplemented group. The drug was administered as a single intravenous bolus at a dose of 10 μg/kg. Partial heart ischaemia was induced by occlusion of the left descending coronary artery. After 3 h ischaemia the dogs were killed, their hearts were removed and the levels of the above-mentioned peroxidation products were assayed. The concentrations of TBARS, conjugated dienes and hydroperoxides were significantly increased not only in the ischaemic region, but also in the border zone and in the "normally" perfused region of the heart. A considerable decrease of these products was observed in the Iloprost-treated group. A direct effect of Iloprost on the heart cell membranes is postulated.

Key words: Heart, ischaemia, peroxidation, Iloprost

The biggest amount of oxygen diffusing into cells undergoes a tetravalent reduction in mitochondria by cytochrome oxidase, producing water (Chance et al., 1979). Five to ten per cent of the oxygen, however, undergoes a monovalent reduction resulting in superoxide anion, hydroxyl radical and hydrogen peroxide (Brunori and Rotilio, 1984).

$$O_2 \xrightarrow{e} O_2^- \xrightarrow{e} O_2^{2-} \xrightarrow{e} O^- \xrightarrow{e} O^{2-}$$

There are several potential sources of oxygen radicals in the cell: xanthine oxidase, aldehyde oxidase, flavin dehydrogenase, peroxidases, cytochrome P-450, haemoglobin and myoglobin, ferredoxin and some others (Fridovich, 1978). The most toxic of them is the hydroxyl radical O^-, as it may react with lipids and other cellular components generating other radicals (Brody et al., 1974). Peroxisomes,

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identified in mouse and cat heart cells (Hand, 1974; Herzog and Fahimi, 1975), include enzymes generating O_2^{2-} such as glycolate oxidase, uric acid oxidase and flavoprotein dehydrogenase, which may take part in the β-oxidation of fatty acids (Connock and Perry, 1983), simultaneously generating hydrogen peroxide. Catalase itself can also catalyse some peroxidase-type reactions in the presence of hydrogen peroxide (Sies, 1977).

Disturbances in mitochondrial electron transport with the release of ubisemiquinone and flavoproteins, the accumulation of cell metabolites such as NADH, lactate and catecholamines, the reactions catalysed by xanthine oxidase (Granger and Parks, 1983), and the acute inflammatory response (Romson et al., 1982) may contribute to the production of free radicals in the infarcted heart. The accumulation of reducing equivalents due to disturbed electron transport in mitochondria (i.e. the excess of electron donors) is accompanied by the appearance of molecular oxygen (Casals et al., 1985). The normal oxygen tension in tissues is 35 mm Hg, while in the ischaemic zone it does not go below 5–10 mm Hg (Demopoulos et al., 1980). Free radical production, reaching one half of that produced under normal conditions, may already occur at a partial oxygen tension of 1 mm Hg (Noll and De Groot, 1984). Moreover, it was shown that free radical induced lipid peroxidation was rapid in brain homogenates incubated in the presence of only 5% O_2 (Smith et al., 1980).

Indirect evidence of free radical involvement in ischaemic injury comes from studies on the protective effect of their scavengers on the hypoxic heart. The infusion of α-tocopherol in hypoxia inhibits the expected consequences of molecular oxygen reintroduction (Guarnieri et al., 1978). This fact, together with data on the positive effect of glucose infusion in similar conditions (Hess et al., 1984), suggests that oxidate reactions are the primary factors causing mechanical and biochemical injury of cardiac cells in the ischaemic phase. The known hydroxyl radical scavenger dimethylsulfoxide, when added to the perfusate, reduced the release of creatine phosphokinase, prevented the formation of contracture zones, and maintained the proper architecture of the myocardium (Ganote et al., 1982). Moreover, this compound prevented changes in mitochondrial activity 2 h after complete heart ischaemia (Schlafer et al., 1982). Mannitol, the other hydroxyl radical scavenger, showed similar activity (Lucas et al., 1980).

Previous works (Ledwożyw, 1991*a,b*) demonstrated a positive salutary effect of free radical scavengers in bleomycin-induced lung fibrosis, which is also free radical dependent.

The most commonly used markers of the intensity of peroxidative processes are the determination of thiobarbituric acid reactive substances (TBARS), conjugated dienes and hydroperoxides (Slater, 1984).

During the search for drugs possessing a possible therapeutic value in the treatment of cardiac infarction, attention was drawn to prostacyclin and its ana-

logues (Blasko et al., 1983). The stable prostacyclin analogue Iloprost (ZK 36374, Schering AG, Berlin, Germany) shows strong antiaggregatory (Cowley et al., 1985) and cardioprotective (Smith et al., 1984) properties.

As the literature is lacking any data concerning the effect of Iloprost on peroxidative processes during the course of myocardial ischaemia, the present paper attempts to examine the influence of this compound on TBARS, conjugated dienes and hydroperoxide production in the dog myocardium and blood plasma after occlusion of the left descending coronary artery.

Materials and methods

The experiment was performed on 25 male mongrel dogs (body mass range: 18.5–25.5 kg). The dogs were divided into 3 groups: (1) sham-operated (thoracotomy and pericardiotomy only; n = 9), (2) control (no drug; n = 8), (3) ZK 36274 supplemented animals (10 mg/kg; n = 8). The drug was administered just before the coronary artery occlusion.

Animals were anaesthetized with intravenous infusion of sodium pentobarbital (30 mg/kg) and Fentanyl (0.05 mg/kg). Surgical anaesthesia was maintained after endotracheal intubation with NO + O 2:1 v/v. Supplemental doses of Fentanyl (0.05 mg/kg) were administered if and when needed to abolish the corneal reflex. Positive pressure was maintained at 16 cycles/min, and tidal volume at 15 ml/kg body mass using a Chirana respirator. Pancuronium (0.1 mg/kg, i.v.) was administered to prevent reflex muscular movement. A teflon cannula with 3-way stopcock was inserted into the left femoral vein for drug administration.

The heart was exposed under sterile conditions through a midsternotomy and, after pericardiotomy, the left anterior descending coronary artery was ligated approx. 15–30 mm distal from the tip of the left atrial appendage. In each case complete occlusion was evidenced by the appearance of a nonpulsatile, nondistended artery, distal to the occlusion and nondistended veins draining from the ischaemic zone, and by the immediate appearance of cyanosis and the bulging of the ischaemic segment. A bolus of 20 ng xylocain was administered i.v. to suppress arrhythmias. At 3 h following the occlusion, the experiment was terminated, the heart was removed, the ligature cut and the heart perfused with cold (4 °C) 0.02 M Tris-HCl buffer, pH = 7.4. Samples were taken from the center of the visible ischaemic area (a), border zone (b) and "normally" perfused region of the posterior wall of the left ventricle (c). In group A (sham-operated dogs), pieces of heart muscle were taken from the whole thickness of the wall in the region parallel to the ischaemic zone in the control group (B) and Iloprost-treated group (C).

The heart muscle was homogenized in 10 vol. of 0.02 M Tris-HCl buffer, pH = 7.4 in an all-glass homogenizer. The homogenate was centrifuged for 10 min at 5000 *g*. The TBARS level was determined in the supernatant as described pre-

viously (Ledwożyw et al., 1986). Conjugated dienes and hydroperoxides were estimated according to Ward et al. (1985). The concentrations of these compounds were determined in the peripheral blood plasma as well.

Statistical significance of the differences was analysed by Student's *t* test for unpaired data.

Results

The concentrations of TBARS, conjugated dienes and hydroperoxides were significantly increased in the ischaemic region of control dogs. The increase, although not so intense, was also observed in the border zone and in the "normally" perfused area of the heart. The intensity of these processes was significantly lower in the Iloprost-treated than in the control group (Table 1).

Table 1

Thiobarbituric acid reactive substances (TBARS) (nM/g), conjugated dienes (optical density at 233 nm/g) and hydroperoxides (optical density at 353 nm/g) in ischaemic area (a), border zone (b) and normally perfused area (c) of dog myocardium after 3-h occlusion of the left anterior descending coronary artery

Group	Areas of dog myocardium	Conjugated dienes	Hydroperoxides	TBARS
Sham-operated dogs (A; n = 9)		0.180 ± 0.020	0.440 ± 0.050	4.20 ± 0.45
Control group (B; n = 8)	ischaemic area (a)	0.650 ± 0.080*	0.900 ± 0.100*	7.18 ± 0.76*
	border zone (b)	0.320 ± 0.040*	0.750 ± 0.080*	5.60 ± 0.58*
	normally perfused area (c)	0.250 ± 0.030*	0.620 ± 0.070*	4.80 ± 0.51*
Iloprost-supplemented group (C; n = 8)	ischaemic area (a)	0.250 ± 0.030*	0.520 ± 0.055*	5.38 ± 0.56*
	border zone (b)	0.230 ± 0.025*	0.480 ± 0.050*	4.62 ± 0.49*
	normally perfused area (c)	0.200 ± 0.020*	0.420 ± 0.040*	4.45 ± 0.47*

*$P < 0.05$ B vs. A and C vs. B

The levels of the above-mentioned peroxidation products were also significantly higher in the peripheral blood of control dogs. A considerable decrease in the levels of these compounds was observed in the Iloprost-supplemented group (Table 2).

Table 2

Thiobarbituric acid reactive substances (TBARS) (μM/l), conjugated dienes (optical density at 233 nm/ml) and hydroperoxides (optical density at 353 nm/ml) in peripheral blood plasma of dogs after 3-h occlusion of the left anterior descending coronary artery

Group	TBARS	Conjugated dienes	Hydroperoxides
Sham-operated dogs (A; n = 9)	1.40 ± 0.18	0.200 ± 0.025	0.080 ± 0.010
Control group (B; n = 8)	4.35 ± 0.50*	0.630 ± 0.070*	0.240 ± 0.030*
Iloprost-supplemented group (C; n = 8)	3.00 ± 0.32*	0.350 ± 0.040*	0.150 ± 0.030*

*P < 0.05 B vs. A and C vs. B

Discussion

Free radical-induced peroxidation of membrane lipids is the most important negative process in the cell (Di Luzio, 1973). Within biomolecules, the unsaturated acyl chains of membrane phospholipids are extremely sensitive to free radical damage due to the following factors: (a) α-methylenic carbon has allylic hydrogen, (b) molecular oxygen is 7 times more soluble in the nonpolar environment than in water, and (c) molecular oxygen has the external orbitals with unpaired electrons, which provide it with some free radical properties, such as the ability to induce sequential free-radical reactions in sensitive molecules (Aust and Svingen, 1982). Membrane lipid injury is a relevant element of free radical toxicity, as most enzymes maintain their active configuration as a result of interaction of the hydrophobic part of the protein molecule with the acyl chains of the membrane phospholipids (Farias et al., 1975). Free radicals may also form new channels in the membranes, conducting molecules and ions (Guskova et al., 1984), especially Ca^{2+} (Lebedev et al., 1982).

The production of hydroxyl radicals in rabbit heart mitochondria subjected to 40 min ischaemia was demonstrated by Otani et al. (1984) and Suzuki (1975) using electron spin resonance spectroscopy. Mitochondrial respiration is a rich source of oxygen radicals. After blocking the electron transport with actinomycin A, an intense production of hydrogen peroxide occurs. The interaction of reduced

CoQ with oxygen also results in the production of oxygen radicals (Boveris, 1977). CoQ readily undergoes autooxidation, especially during its separation from other elements of electron transport. In studies on the conditions of TBARS production during NADH-dependent oxidation in bovine submitochondrial particles, it was demonstrated that the intensity of membrane lipid peroxidation increases during CoQ oxidation (Takayanagi et al., 1980). Using a rat and a dog model of heart ischaemia, Rao and Mueller (1982) showed an increase in peroxidation products (malondialdehyde, H_2O_2) and an increased level of free radicals (electron spin resonance spectroscopy) and carbon-centered radicals. Majewska et al. (1978) have observed an increased production of malondialdehyde in guinea pig brain mitochondria shortly after the onset of ischaemia.

The present paper showed an increase in the level of peroxidation products in the ischaemic zone of the dog heart 3 h after the occlusion of the left descending coronary artery. This confirms the data presented above, suggesting a causal involvement of free radicals in the ischaemic injury of the myocardium. An increased amount of these compounds was also observed in the border zone between the ischaemic and the "normally" perfused region.

Most probably, the activation of peroxidative processes in the border zone is the result of a natural reoxygenation caused by reactive hyperaemia and collateral blood flow. It is most likely that this phenomenon is responsible for the spreading of the necrotic zone during a myocardial infarct. Twenty four h after coronary artery occlusion, Dillard and Tappel (1971) observed a 3.5 times higher amount of conjugated dienes in the ischaemic focus and twice as much in the border zone. The content of fluorescent Schiff's bases was 13 times higher in the ischaemic zone and 5 times higher in the border zone. Thermoluminescence reached its maximum 3 days after coronary artery ligation and gradually decreased up to the 30th day (Meerson et al., 1982). The present work also showed an intensive increase of peroxidative processes in the "normally" perfused region of the heart (posterior wall of the left ventricle). This may be caused either by free radical diffusion from the ischaemic and border zone, or by a phenomenon similar to the "oxygen paradox" (Post et al., 1985) taking place in the reactive hyperaemic zone of the myocardium. Free radical overproduction may also be caused by the activation of polymorphonuclear leukocytes accumulating in the necrotic zone (Johnson et al., 1980). The observed increase in the level of peroxidation products in the "normally" perfused region is also likely to be caused by the free radical reactions occurring in the capillary endothelium. The oxidation of arachidonic acid to PGG_2 in platelets is a free radical mediated mechanism (Marnett, 1984). PGG_2 is then converted into PGH_2 with an accompanying release of the peroxide radical (Prosdocimi et al., 1985). As a result, TXA_2 is produced, promoting platelet aggregation and adhesion to endothelium (Hamberg et al., 1976). The ability of the vessel wall to produce PGI_2 neutralizing the biological effects of thromboxane is significantly decreased in the presence of lipid peroxides (Lands et al., 1984), and

that decrease is far quicker than that of their ability to produce TXA_2 (Bourgain et al., 1982). Thus, the relative thromboxane overproduction probably leads to the formation of microthrombi in capillaries of the "normally" perfused zone and, consequently, to expansion of the injury. This hypothesis is confirmed by the fact that 1–3 h after brain artery occlusion in the cat, an intensive adhesion of platelets and leukocytes to the endothelium was observed (Demopoulos et al., 1980). If the increase in prostaglandin synthesis is a rapid process, then it is possible that the production of free radicals may exceed the protective ability of the tissue (Wei et al., 1981). Thus, the results of this work suggest that the increased production of free radicals after occlusion of the left descending coronary artery in the dog is not limited to the ischaemic zone but extends to the whole myocardium, and the biochemical changes are more extensive than the size of the ischaemic region would suggest.

The present work has revealed that Iloprost significantly decreased the amount of TBARS, conjugated dienes and hydroperoxides in the ischaemic myocardium of the dog. The mechanism of this phenomenon is difficult to explain. It may consist of a decrease in thromboxane production inhibiting the activation and aggregation of platelets, a decrease in the oxygen requirement of the myocardium, or a direct protective action on cell membranes (Muller et al., 1986). However, the direct effect of Iloprost on the cell membrane remains an interesting hypothesis, since a cytoprotective effect of prostaglandins including prostacyclin on erythrocytes (Ledwożyw et al., 1985; Ledwożyw and Kądziołka, 1986; Michalak et al., 1986), gastric mucosa cells (Robert et al., 1979) and hepatocytes (Újhelyi et al., 1984) has been shown. Thus, it is probable that Iloprost acts in a similar manner, by stabilizing the membranes of cardiac cells.

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LACK OF ACID-RESISTANT TRYPSIN INHIBITOR IN MARE'S COLOSTRUM: SHORT COMMUNICATION

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Mare's colostrum was collected and examined for the presence of trypsin inhibitors. It was found to contain a low level of trypsin inhibitor which could be denatured by 2.5% trichloroacetic acid and, therefore, it clearly differs from the acid-resistant colostral inhibitor of Artiodactyla and Carnivora. This finding is exceptional for a species that concentrates IgG in the colostrum and whose newborn absorbs colostral proteins non-selectively by the gut. It appears that the presence of colostral trypsin inhibitor is not essential for the transmission of maternal immunity via the colostrum and the gut.

Key words: Horse, colostrum, trypsin inhibitor, immune transmission, IgG, newborn, Artiodactyla, Perissodactyla, Carnivora, Ungulata

The cow's colostrum contains a trypsin inhibitor which is resistant to 2.5% trichloroacetic acid (Laskowski and Laskowski, 1951). A similar inhibitor was found in the colostrum of the sow (Laskowski et al., 1957) and several species of Carnivora (Baintner, 1984). The low level of trypsin inhibitor in the human colostrum is not acid-resistant and appears to be a mixture of serum-derived inhibitors (Barkholt-Pedersen et al., 1971).

In Artiodactyla the placenta does not transport maternal antibodies to the fetus. Maternal immunity, essential for extrauterine life, is acquired by the intestinal absorption of intact immunoglobulins of the ingested colostrum during the first 1 or 2 days of extrauterine life (reviewed by Brambell, 1970, and Baintner, 1986). A similar process takes place in at least one species of Perissodactyla, in the newborn foal (Rumbaugh et al., 1979; Galan et al., 1986) and in several species of Carnivora, although the latter also acquire some immunity prenatally. In the above-mentioned taxonomic groups the newborn absorbs colostral proteins with little or no selection between immunoglobulins and other milk proteins, whereas the absorption of IgG in the postnatal rat and mice is a highly selective process. It was shown (Baintner, 1986) that three characteristics tend to go concomitantly: (1) non-selective absorption of colostral proteins (detected immunologically or by the characteristic histological appearance of the absorptive cells); (2) IgG domi-

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nance in the colostrum; and (3) presence of acid-resistant ("colostral") trypsin inhibitor. However, the present experiments show that the horse (*Equus caballus*, Perissodactyla) may be an exception.

Materials and methods

Colostrum samples were collected from 16 Hungarian draft horse mares within 10 h after parturition ($\bar{x}$ = 3.5 h), including 6 samples taken immediately after parturition, and stored in deep freeze until use.

Fat was removed from the samples by centrifugation and an aliquot of the subnate was diluted twofold with physiological NaCl solution. Then the liquor was mixed with the same volume of 5% trichloroacetic acid (TCA, 2.5% final concentration) and left standing at 4 °C for 1 h. Precipitated proteins were removed by centrifugation and the supernate was neutralized with the same volume of 0.1 M Tris-HCl buffer, pH 8. A parallel series of aliquots was diluted 8-fold with physiological saline to match the final dilution of the TCA-treated series. Trypsin inhibitor was determined in both series of treatments according to the UV spectrophotometric method of Schwert and Takenaka (1955), with N-α-benzoyl-L-arginine ethyl ester (BAEE) as substrate. The amount of inhibitor was calculated as trypsin quantity inhibited by one ml of sample.

Results and discussion

A low level of trypsin inhibitor was measured in the colostrum samples: 1.45 ± 0.6 mg/ml (mean ± standard deviation). The values showed a non-significant tendency to decrease with increasing sampling time. In the TCA-treated samples no inhibitor was detected, which shows the lack of acid-resistant inhibitor in mare's colostrum.

Ungulates (Artiodactyla and Perissodactyla) concentrate IgG in the colostrum, which is absorbed non-selectively by the newborn gut. Colostral trypsin inhibitor (CTI) provides some protection for the IgG from being digested before or during absorption. Other contributing factors may be the sluggish development of gastric and pancreatic secretion and the quantity of the ingested colostrum. It appears, however, that the presence of CTI is not essential for the transmission of maternal immunity via the colostrum and the gut. The results also show a biochemical difference between Artiodactyla and at least one species of Perissodactyla, the horse.

In the multiparous Carnivora and pig the higher level of CTI provides a better protection of colostral antibody in the gut for the weakest members of the

litter, who cannot suckle much colostrum because of the more competitive littermates, as compared to the uniparous species, i.e. the horse (no CTI) and ruminants (low CTI level), where the selective pressure might be smaller.

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EFFECT OF VITAMINS A AND E ON BLOOD PLASMA VITAMIN STATUS AND DAILY BODY MASS GAIN OF DIFFERENT FAT-TAILED SHEEP BREEDS

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Ninety ram lambs of three Iranian fat-tailed sheep breeds including the Shal breed (age: 4–6 months) in one experiment and the Sanjabi and Bakhtiari breeds (6–9 months) in another experiment were assigned to five groups of 5–7 lambs each to receive a basal diet plus different levels of vitamins A and E supplementation. The groups received 0, 50 or 100 IU vitamin A/kg body mass (b.m.) and 0, 15 or 30 IU vitamin E/kg of diet. In the vitamin A groups a constant level of 15 IU vitamin E and in the vitamin E groups a constant level of 50 IU vitamin A supplementation was provided. Blood plasma samples were taken at the beginning and at the end of the experiments and the concentrations of vitamins A and E were determined by high-performance liquid chromatography (HPLC). Vitamin A supplementation of the highest level (100 IU/kg b.m.) significantly increased the retinol content of the blood plasma in the Shal ($P < 0.01$) and Bakhtiari ($P < 0.05$) breeds. The mild increase of retinol concentration in the Sanjabi breed was not significant ($P > 0.05$). Vitamin A supplementation greatly decreased the α-tocopherol content of the blood plasma in all three breeds ($P < 0.01$). It increased the average daily body mass gain of lambs of the Shal breed ($P < 0.01$) but decreased that of Bakhtiari lambs ($P < 0.05$). Supplementation of vitamin E mildly decreased the retinol content of the blood plasma in all the breeds and the decrease was statistically significant ($P < 0.05$) for the Shal breed at the highest dose level. Vitamin E supplementation greatly increased the α-tocopherol concentration of the blood plasma in all the breeds ($P < 0.01$–0.001). It decreased the average daily body mass gain of the Shal breed ($P < 0.001$) but improved it in the two other breeds. That increase was statistically significant ($P < 0.05$) in the Bakhtiari breed. It was concluded that the Shal breed gave a better response to vitamin A supplementation while the Sanjabi and Bakhtiari breeds to vitamin E. This indicates that fat-tailed breeds have dissimilar requirements for vitamins A and E supplementation. A higher level of vitamin E intake is required for raising the α-tocopherol content of the blood plasma above the level critical for sheep (0.8 μg/ml).

Key words: Fat-tailed sheep, retinol, tocopherol, body mass gain

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Vitamin A was the first liposoluble vitamin to be discovered and characterized. It has essential functions in vision, bone and muscle growth, reproduction and the maintenance of healthy epithelial tissues (National Research Council, 1987). Vitamin E was also recognized about 70 years ago (Evans and Bishop, 1922). It is required for the antioxidative protection of cells and its various functions still remain to be identified. Vitamin A metabolism has been linked with vitamin E because of the antioxidative properties in the stability of biological membranes. Interrelationships of vitamin A and vitamin E have been reported from laboratory animals (Moore, 1940; Davies and Moore, 1941; Irving, 1958; Yang and Desai, 1977). Such interrelationships have also been described for ruminants (Rousseau et al., 1957; Dicks et al., 1959; Hidiroglou and Williams, 1986). However, the results reported on their interaction are rather contradictory. This contradiction may be due to differences in species, dosage level and trial length. Early reports showed a beneficial effect of vitamin E on the body mass gain of calves (Reddy et al., 1985; Reddy et al., 1987; Pehrson et al., 1991). Jenkins and Mitchell (1975) found that increasing vitamin E levels had a positive while increasing vitamin A levels exerted a negative influence on the growth of rats.

The objective of this work was to study the effect of different levels of vitamins A and E on blood plasma vitamin content and on the interactions of these vitamins in the blood plasma. A further objective was to determine the effect of the vitamins on the average daily gain (ADG) of three breeds of fat-tailed sheep during the fattening period.

Materials and methods

Animals and treatments

Experiment 1. Twenty-eight 4- to 6-month-old ram lambs of an Iranian fat-tailed sheep breed named Shal were used. The lambs were purchased from the place where the breed is usually raised, the Shal region. They were divided into 5 groups of 5–6 sheep each. The groups received a basal diet plus 3 levels of vitamins A or E supplementation. The levels for vitamin A were 0 (A_0), 50 (A_1) and 100 (A_2) IU/kg body mass (b.m.) (Rovimix A-500, Hoffman-La Roche Ltd., Basle). The levels for vitamin E were 0 (E_0), 15 (E_1) and 30 (E_2) IU/kg diet (Rovimix E-50 Adsorbate, Hoffmann-La Roche Ltd., Basle). All vitamin A groups including the control received a constant amount of vitamin E supplement (15 IU/kg diet). All vitamin E groups were given a constant amount of vitamin A supplement (50 IU/kg b.m.). The supplements were added to 35 grams of fresh wheat straw per head and used prior to daily feeding. Lambs had an initial average body mass of 35.2 ± 0.9 kg (mean ± SD) and were fattened for 95 days. They were fed 0.5 kg wheat straw and 1.18 kg of a concentrate composed of 60% unground barley, 23.8% wheat bran, 15% cottonseed meal and 1.2% calcium car-

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bonate for the first 25 days. In the remaining period they were fed the same amount of wheat straw plus 1.3 kg concentrate feed composed of 60 % unground barley, 28.8% wheat bran, 10% cottonseed meal and 1.2% calcium carbonate. The crude protein content of the first concentrate was 14.8 ±0.4% DM and that of the second one 13.3 ± 0.3% DM.

Experiment 2. Sixty-two 6- to 9-month-old ram lambs of two Iranian fat-tailed sheep breeds named the Sanjabi (n = 32) and the Bakhtiari (n = 30) breed were used. The lambs were donated by the National Organization of Meat (Ziaran Devisin, Tehran) and they were fattened under the same conditions. They were divided into 5 groups of 5–7 sheep each. The groups received three levels of vitamins A and E supplementation, as described in experiment 1. Lambs of the Sanjabi breed had an initial average body mass of 40.8 ± 0.9 kg and those of the Bakhtiari breed 41.4 ± 0.9 kg. The fattening period was 94 days for both breeds. All the groups were fed 0.5 kg wheat straw and 1.3 kg concentrate of the same composition as in the second ration of experiment 1. The crude protein content was 13.5 ± 0.4%.

All the animals in the two experiments were raised together for about 2 months. They received appropriate vaccines and were dewormed and sheared before the beginning of the experiments. The lambs had free access to water and salt. Their body mass gain was recorded every third week. At the beginning and at the end of the experiments blood samples (5 samples/group) were taken from the jugular vein into EDTA-containing tubes. The plasma was separated and stored in air-tight polypropylene vials at –20 °C until assayed for vitamin A and E concentration.

Samples from the feed were taken every 4 weeks (5 samples) for β-carotene, α-tocopherol and crude protein determination. At the end of the two experiments, all the lambs were slaughtered and the carcasses kept at 3–4 °C. The dressing percentage and the tail fat to carcass weight ratio were also calculated.

Analytical methods

Quantitation of retinol and α-tocopherol concentrations in the blood plasma of lambs was done by a high-performance liquid chromatography (HPLC) instrument with an UV detector. For each sample parallel quantitations were carried out. Precipitation of plasma (1 ml) protein with absolute ethanol (1 ml, containing 0.125% BHT) and extraction with n-hexane, twice (5 ml, containing 0.025% BHT) were done according to the method of Biesalski et al. (1986) and antioxidant application as described by Chow and Omaye (1983).

The extract was dried under nitrogen stream and the residue was dissolved in 0.5 ml methanol. The solvent was vortexed and sonicated for 1 min, then centrifuged for 15 min with 8000 *g*. An amount of 50 µl was injected into a Varian

5000 HPLC instrument equipped with a reverse-phase column (Nucleosil C18, 250 × 4 mm, 5 μm particle size, Bio-Separation Technologies Co., Budapest).

A common wavelength of 292 nm was chosen for the determination of retinol and α-tocopherol. The eluent was methanol/water (97:3) with a flow rate of 1.5 ml/min.

Retinol and dl-α-tocopherol standards were used for peak identification (Fluka Chemica-BioChemika, Bucks). Identification and quantitation of the vitamins were accomplished by comparison of retention time and peak area. Determination of α-tocopherol in the feed was based on the method of McMurray and Blanchflower (1979), while total carotene was determined by a spectrophotometer (Spectronic 20, Bausch and Lomb, Rochester, New York) according to the method of the A.O.A.C. (1990).

Mathematical analysis

Analysis of variance based on a completely randomized design and the least significant difference method (Student's *t* test) and regression analysis were used for group comparisons.

Results

The concentration of α-tocopherol in the first and the second concentrate was 16.1 ± 0.8 and 16.4 ± 0.7 mg/kg DM, respectively. The amount of β-carotene was 2.25 ± 0.45 mg and 2.30 ± 0.55 mg/kg DM in the first and second concentrate, respectively.

Analysis of variance showed that vitamin A supplementation had a significant effect on the average daily gain (ADG) of all the breeds ($P < 0.01$) and that the breeds' response to different levels of vitamin A supplementation was significantly different ($P < 0.001$). The results also indicated that different levels of vitamin E supplementation exert a dissimilar effect on the ADG of each breed ($P < 0.05$). Yet, the breeds probably give dissimilar ADG responses to different levels of vitamin E ($P < 0.09$).

Experiment 1

The retinol content of the plasma was increased and influenced by vitamin A supplementation ($P < 0.05$). The response was highly significant ($P < 0.01$) at the highest dose level (100 IU/kg b.m.). The zero level of vitamin A supplementation led to a significant ($P < 0.05$) decrease in retinol content. Vitamin E supplementation at 0 or 15 IU/kg of diet did not decrease the plasma retinol concentration significantly. However, the highest level (30 IU) of vitamin E significantly ($P < 0.05$) reduced the retinol content.

The α-tocopherol content was greatly decreased ($P < 0.001$) by vitamin A supplementation. The decrease was negatively correlated with the second ($P < 0.01$) and third ($P < 0.001$) dose level (50 and 100 IU/kg b.m.). At the same time, vitamin E supplementation increased α-tocopherol content of the blood plasma at the highest dose level (30 IU/kg diet).

Vitamin A supplementation significantly ($P < 0.01$) increased while vitamin E supplementation decreased ($P < 0.001$) the ADG in the Shal breed.

The dressing percentage was 47.6%, the weight of tail fat 4.5 ± 0.3 kg and the average ratio of tail fat to carcass weight was 18.2%. The ADG for this breed was 185 ± 5 grams.

Table 1

Effect of vitamins A and E supplementation on the content of retinol and α-tocopherol in the blood plasma and on the average daily body mass gain of fat-tailed sheep of the Shal breed (mean ± SD)

Vitamin A intake, IU/kg b.m.	Vitamin E intake, IU/kg of diet (DM)	Average daily gain (g) (n=5–6)	Retinol, mg/L (n=5)	R^3	α-tocopherol mg/L (n=5)	R^4
0	15	$159.6\pm4.7^{b,t}$	$0.45\pm0.01^{c,i}$	$0.64^{b,t}$	$1.13\pm0.05^{a,i}$	$-0.78^{a,t}$
50	15	175.5 ± 1.4^{c}	0.48 ± 0.02	0.49	0.84 ± 0.04^{b}	-0.85^{b}
100	15	186.0 ± 6.2^{b}	0.49 ± 0.00^{b}	0.85^{b}	0.79 ± 0.04^{a}	-0.88^{a}
50	0	$198.7\pm2.6^{a,t}$	0.48 ± 0.01	–0.50	$0.72\pm0.06^{b,}$	$+0.77^{b,t}$
50	15	175.5 ± 1.4^{a}	0.48 ± 0.02	–0. 04	0.84 ± 0.04	+0.47
50	30	182.5 ± 4.2^{b}	0.44 ± 0.01^{c}	-0.73^{c}	1.07 ± 0.07^{a}	$+0.82^{b}$
Initial value		–	0.48 ± 0.01	–	0.76 ± 0.03	–

a: significant at $P < 0.001$; b: $P < 0.01$; c: $P < 0.05$; t: analysis comparing all groups; i: analysis comparing the group with initial values; R^3 and R^4 : regression coefficients between intake vs. blood levels

Experiment 2

Sanjabi breed: The retinol content of the blood plasma was slightly increased by vitamin A supplementation; however, the differences were not statistically significant. Vitamin E supplementation decreased the retinol content of the blood plasma but the differences were not significant either ($P > 0.05$). The α-tocopherol content decreased significantly ($P < 0.001$) as an effect of vitamin A and increased with vitamin E supplementation ($P < 0.001$). Regression analysis showed a more expressed effect ($P < 0.001$) at the highest dose level as compared to the medium level (15 IU/kg diet) of vitamin E application ($P < 0.01$). In addi-

tion, vitamin E supplementation (without vitamin A) produced a highly significant increase in α-tocopherol content as compared to the initial values ($P < 0.001$).

The Sanjabi breed showed a lower ADG negatively correlated with increasing vitamin A supplementation. However, the values were not significantly different ($P > 0.05$). The decrease of ADG was 3.5% at the second dose level (50 IU/kg b.m.) and 8.4% at the third dose level of vitamin A administration (100 IU/kg b.m.). Vitamin E supplementation increased the values of ADG in the Sanjabi breed. The increase was 3.6% at the second (15 IU/ kg diet) and 8.3% at the third level (30 IU/kg diet) of application. The best result was observed when the ratio of vitamin E was higher as compared to vitamin A.

The average dressing percentage was 51.4%, the weight of tail fat 6.1 ± 0.3 kg, and the ratio of tail fat to carcass weight 21.3%. The ADG for this breed was 176 ± 4 grams.

Bakhtiari (Louri) breed: Vitamin A supplementation increased the retinol content of the blood plasma. This effect, however, was significant ($P < 0.05$) and positively correlated only at the highest dose. The lack of vitamin A supplementation greatly decreased the retinol content of the blood plasma ($P < 0.001$). The administration of vitamin E slightly decreased the retinol concentration but the effect was not significant ($P > 0.05$). The concentrations of α-tocopherol markedly decreased in sheep given vitamin A supplements ($P < 0.001$). Vitamin E supplementation increased the concentrations of α-tocopherol ($P < 0.001$) in the blood plasma.

Supplementation of vitamin A decreased the ADG in this breed ($P < 0.05$). General regression analysis showed a negative correlation between ADG and different levels of vitamin A ($P < 0.01$). In contrast, raising the vitamin E levels increased the ADG. The observed differences were not significant but regression analysis showed positive correlations between all levels of vitamin E and the ADG ($P < 0.05$). In this breed, the blood plasma concentration of tocopherol was the highest when vitamin E was fed at 30 IU/kg of diet. The differences were significant ($P < 0.05$) as compared to the Shal breed.

The ADG decreased by 10.9% in sheep receiving vitamin A at 100 IU/kg b.m. and increased by 6.4% at the highest level of vitamin E supplementation (30 IU/kg of diet).

The average dressing percentage was 51.1%, the weight of tail fat 6.4 ± 0.4 kg, and the ratio of tail fat to carcass weight was 21.2%. The ADG of this breed was 186 ± 4 grams.

The breeds did not show differences in the initial values of plasma retinol content. Vitamins A and E supplementation did not induce significant differences in retinol concentrations between the breeds at any level of supplementation. How-

ever, the highest dose level of vitamins A and E (A_2 and E_2) produced significant ($P < 0.05–0.01$) differences between the Shal and the Sanjabi breeds.

Comparison of the initial values of tocopherol concentration and the results obtained after the highest level of vitamin A supplementation showed significant differences among and between groups. There were statistically significant differences between the breeds in initial values of α-tocopherol in the blood plasma. The differences were significant ($P < 0.001$) for Shal vs. Sanjabi and Shal vs. Bakhtiari breeds ($P < 0.01$). Only the highest level of vitamin E supplementation caused a significant difference ($P < 0.05$) in α-tocopherol concentration between the Shal and the Bakhtiari breeds.

Table 2

Effect of the supplementation of vitamins A and E on the blood plasma concentrations of retinol and α-tocopherol and on the ADG of fat-tailed sheep of the breeds Sanjabi and Bakhtiari (mean ± SD)

Vitamin A intake, IU/kg b.m.	Vitamin E intake, IU/kg of diet (DM)	Average daily gain (g) (n=5–7)	Retinol, mg/L (n=5)	R^3	α-tocopherol mg/L (n=5)	R^4
			Sanjabi breed			
0	15	189.4±8.5	$0.45±0.00^{b,i}$	0.40	$1.28±0.08^{a,i}$	$-0.93^{a,t}$
50	15	182.8±8.8	$0.46±0.01^{c}$	0.48	$0.87±0.07^{b}$	-0.81^{b}
100	15	173.5±4.6	$0.46±0.01^{b}$	0.56	$0.51±0.03^{a}$	-0.96^{a}
50	0	176.4±5.5	0.47±0.01	–0.06	$0.55±0.05^{a,t}$	$+0.87^{a,t}$
50	15	182.8±8.8	0.46±0.01	–0. 07	$0.87±0.07^{b}$	$+0.78^{b}$
50	30	191.0±8.7	0.46±0.01	–0.07	$1.13±0.07^{a}$	$+0.92^{a}$
Initial value		–	0.48±0.01	–	0.53±0.03	–
			Bakhtiari breed			
0	15	$202.7±5.9^{c,t}$	$0.44±0.01^{a,i}$	$0.64^{b,t}$	$0.91±0.07^{a,t}$	$-0.84^{a,t}$
50	15	$183.7±3.1^{c}$	0.45±0.01	0.36	$0.73±0.04^{c}$	-0.64^{c}
100	15	$180.7±5.3^{c}$	$0.48±0.01^{c}$	0.69^{c}	$0.55±0.04^{a}$	-0.86^{a}
50	0	179.7±5.4	$0.46±0.00^{a,i}$	–0.36	$0.42±0.02^{a,t}$	$+0.96^{a,t}$
50	15	183.7±3.1	0.45±0.01	–0.25	$0.73±0.04^{a}$	$+0.93^{a}$
50	30	191.2±2.8	0.45±0.01	–0.54	$1.30±0.07^{a}$	$+0.97^{a}$
Initial value		–	0.49±0.00	–	0.46±0.03	–

a: significant at $P < 0.001$; b: $P < 0.01$; c: $P < 0.05$; t: analysis comparing all groups; i: analysis comparing the group with initial values; R^3 and R^4 : regression coefficients between intake vs. blood levels

Discussion

Three fat-tailed sheep breeds were used in this study. The Shal breed, which was used in the first experiment, was generally kept under relatively good feeding conditions. Compared to breeds used in experiment 2, the Shal breed is susceptible to a shortage of feed and to diseases. It is characterized by high performance and a smaller fat tail than the other two breeds. The Shanjabi and the Bakhtiari breeds used in experiment 2 have lower production values and less sensitive genotypes as compared to the Shal breed. The Bakhtiari breed had been used and was preferred by nomadic people.

Blood plasma retinol and α-tocopherol

It was found that vitamin A supplementation exerted a more pronounced effect on the α-tocopherol content of the blood plasma than did vitamin E supplementation on the blood plasma retinol content. That indirect effect is well known: e.g. Pudelkiewicz et al. (1964) and Green et al. (1967) reported that excessive doses of dietary vitamin A depressed the apparent absorption of dietary vitamin E.

The results of this study showed that vitamin A supplementation moderately increased the blood plasma retinol content. It is well known that sheep can maintain their plasma retinol concentration based on their feeding condition before the growing and fattening period and their reservoirs. The liver, acting as the main site of storage, plays a major role in vitamin A homeostasis. It regulates the output of retinol to tissues by storing the newly absorbed retinyl esters and releasing retinol in combination with retinol binding protein into the plasma (Goodman et al., 1965). Sheep have a 3–4 times higher concentration of vitamin A in liver tissue than cattle (Moore, 1969; Yang et al., 1992). The results indicate that vitamin A and carotene deficiency in the diet reduces plasma retinol concentration after a long period of time.

Our study showed that the vitamin E content of the basal diet was not sufficient to increase the α-tocopherol concentration of the blood plasma. In contrast, the results demonstrate close positive correlations between the intake of vitamin E and its levels in the blood plasma of lambs, which is consistent with the findings of Bieri (1972) and Yang and Desai (1977). Thus, α-tocopherol concentration of the blood plasma is a good indicator of dietary vitamin E content. The continuous supplementation of vitamin E also seems to be much more important for sheep than that of vitamin A. It is suggested that the proper dose rate of vitamin E, which ensures the critical blood plasma level necessary for preventing possible problems (i.e. nutritional muscular dystrophy, NMD disease) should be determined. It was reported (McMurray and Rice, 1982; Pehrson et al., 1986) that animals with blood levels of less than 1.0–1.5 mg/L α-tocopherol may develop NMD. Hidiroglou et al. (1970) reported that 0.80 mg/L α-tocopherol in the blood

plasma is the critical value for sheep. In this study, the recommended level of vitamin E supplementation (National Research Council, 1985) failed to increase the level of vitamin E over the critical value. However, the use of a vitamin E supplement without vitamin A (A_0) also increased the α-tocopherol concentration of the blood plasma because of the higher ratio of vitamin E used.

Bedő et al. (1992) have reported that there are genetically determined differences in the plasma concentrations of vitamins A and E among different sheep genotypes kept under identical feeding conditions. The response of different breeds may be of dissimilar type and degree. In this study, the Sanjabi breed did not respond to vitamin A supplementation as compared to the other two breeds as regards plasma retinol content. The Shal breed, as the genotype characterized by the highest performance, showed a significant ($P < 0.05$) decrease in plasma retinol content after a high intake of vitamin E (E_2) however, the other two breeds failed to do so. In the Shal breed, vitamin E supplementation hardly increased the α-tocopherol content of the blood plasma, as compared to the other breeds. The Shal breed gave a better response to vitamin A supplementation while the Sanjabi and the Bakhtiari breeds to vitamin E. This can be a result of different requirements that may stimulate reactions affecting absorption, storage and mobilisation.

Growth

Using vitamin A supplementation, Faruque and Walker (1970) observed an increased liveweight gain of milk-fed lambs. Hale et al. (1962) showed that vitamin A increases the performance of steers. However, a depression of growth and feed intake was also reported (Grey et al., 1964) for cattle given high amounts of vitamin A. Our results showed that the ADG of the Shal breed ($P < 0.01$) improved when vitamin A supplementation was increased. On the other hand, that breed gave a negative response to an increasing vitamin E supplementation ($P < 0.001$). The different responses could result from an interaction between the requirements of this breed for vitamins E and A in the phase of growth.

In this study, an increased amount of vitamin E was found to stimulate the growth of the breeds Bakhtiari and Sanjabi. The increase was not statistically significant. However, in the Bakhtiari breed the highest level of vitamin E intake (E_2) showed a significant correlation ($P < 0.05$) with the ADG. The Sanjabi breed failed to show this significant correlation, probably due to the lower rate of ADG. In rats, increasing vitamin E intake was reported to increase the food intake (Jenkins and Mitchell, 1975). The results of Pehrson et al. (1991) showed that vitamin E supplementation in excess of the recommendations of the Agricultural Research Council (1982) increased the ADG of beef cattle and shortened the fattening period. This finding was attributed to the low availability of vitamin E. The reports of Reddy et al. (1985, 1987), Lee et al. (1985) and Hicks (1985) also showed an improved ADG and feed conversion efficiency for young cattle as a

result of additional vitamin E intake. Further experiments are needed to prove the beneficial effect of vitamin E on the performance of growing-finishing lambs of different breeds. It is suggested that in breeds with a higher percentage of tail fat, additional vitamin E intake can improve the body mass gain.

The period of growth and fattening should also be studied to enable a better application of these two vitamins. Vitamin E supplementation is likely to be more critical at the end of fattening, as in that period fat deposition may be dominant and because the adipose tissue may require more tocopherol to resist lipid peroxidation processes. Adipose tissue has been shown to be a preferential tissue for vitamin E storage in sheep (Hidiroglou, 1986; Hidiroglou and McDowell, 1987). Thus, the tail fat tissue may provide a store of vitamin E during the shortage of feed resources (in the winter season).

It can be concluded that (1) a high amount of vitamin E is recommended to elevate the concentration of α-tocopherol in the blood plasma of fat-tailed sheep breeds; (2) the concentration of α-tocopherol is a good indicator of vitamin E intake; (3) different breeds of fat-tailed sheep have dissimilar requirements for vitamins A and E and these differences are based on the production traits of the genotype; and (4) the stage of growth and fattening is a period when correct administration of the vitamins is of increased importance.

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THERAPEUTIC EFFICACY OF POVIDONE-IODINE (BETADINE) AND DICHLOROXYLENOL (SEPTOCID) IN HOLSTEIN COWS AFFECTED WITH ENDOMETRITIS AND/OR CERVICITIS

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The efficacy of either povidone-iodine (Betadine) or dichloroxylenol (Septocid) intrauterine infusions on the treatment of endometritis and/or cervicitis in cows was examined. One hundred and twelve repeat-breeder Holstein cows (aged 3–7 years) were selected for this study. Rectal and vaginal examinations were applied to detect the signs of endometritis and/or cervicitis. Cows were assigned into two groups: the first group (n = 60) was treated with Betadine solution (0.5%) while the second group (n = 52) was given Septocid (0.1%) administered as intrauterine infusion (100–150 ml). Both treatments were repeated at least two times at 7-day intervals and the cows were rechecked. The responding animals were inseminated 10–12 h after oestrus detection using frozen semen from bulls of proven fertility. The success or failure of treatment was evaluated on the basis of the post-treatment conception rate. The relationship of the body weight of cows, the length of the service period (open days) and the number of previous services with the results of treatment with Betadine or Septocid is discussed. The recovery and conception rates obtained after Betadine treatment were better than those obtained after Septocid. Moreover, healthy cows (≥ 500 kg body weight) and those inseminated before post-partum day 180, having no more than 4–7 previous services, responded well to either Betadine or Septocid treatment.

Key words: Endometritis, cervicitis, Holstein cows, povidone-iodine (Betadine), dichloroxylenol (Septocid), intrauterine infusion

Infertility is a major problem in the bovine industry, causing substantial economic losses by reducing the yearly calf production as well as the milk yield.

The early post-partum weeks are stressful for the dairy cow and can be accompanied by several metabolic and infectious conditions (Bretzlaff et al., 1982). Many bovine uteri become infected at the time of parturition and shortly thereaf-

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ter. In most cows, these infections are eliminated rapidly, usually at the first or second oestrus. Ninety to one hundred per cent of dairy cows have a uterine infection during the first 10 to 15 days post partum. This percentage declines to 30% by 30–40 days and to 10–20% by 60 days post partum (Griffin et al., 1974; Hartigan, 1978). The most common lesions found in bovine endometritis were associated with exudation. Due to the abnormal endometrium and uterine infection the early embryo may die and the cow comes into heat again. Repeated services and conception failures may be common symptoms of endometritis (Roberts, 1986).

Most cases of cervicitis originate at the time of, or following, parturition and are often associated with metritis. If severe cervicitis is present, endometritis may also occur. In the bovine species, Pattabi Raman and Bawa (1977) found that the incidence of metritis, pyometra and cervicitis/vaginitis was 29.1, 11.0 and 6.5%, respectively.

Various treatment regimens have been proposed to control infectious problems in the uterus and cervix. Most treatments are based on either promoting oestrous cycling and/or overcoming the suspected or known intrauterine infection. The treatments based on stimulation of uterine muscle tone, blood supply, and flow of mucus are varied. Intrauterine infusions of antibiotics such as streptopenicillin (Khan et al., 1991; Bhaumik et al., 1992), ampicillin (Varshney et al., 1987; Khan et al., 1991), tetracycline and oxytetracycline (Umid Singh et al., 1987, 1988; Khan et al., 1991), neomycin and chloramphenicol (Khan et al., 1991) have been used. Intrauterine infusion with various disinfectants is a relatively common non-antibiotic alternative for the treatment of post-partum infections (Zhang and Chen, 1989; Uppal et al., 1991; Ambrose and Pattabiraman, 1993). Intrauterine irrigation with iodine or Lugol's iodine is the oldest method used for the treatment of endometritis (Anacker, 1879; Albrechtsen, 1917).

The purpose of this research was to determine the efficacy of Betadine and Septocid for the treatment of endometritis and cervicitis and to study the conception rate achieved thereafter.

Materials and methods

This study was conducted on Holstein cows kept on a farm near Giza, Egypt during the period between April and June. The cows were judged to be well-managed and yielded, on average, 20 litres of milk/day. The feeding programme was examined and found to be adequate according to the recommended standards (NRC, 1989). All animals were recorded in a good recording system consisting of cow identification, calving dates, results and date of genital examination, breeding date, sire identification and pregnancy examination results.

Repeat-breeder cows were identified as those which were mated or inseminated three or more times but failed to conceive throughout the lactation period.

Based upon these criteria, 112 cows (aged between 3 and 7 years) were selected for this study. These cows were examined (transrectally and vaginally) to determine the gross characteristics of the genital tract. The experimental animals were apparently healthy and were classified according to their body weight (Rodostits and Blood, 1985) into 3 categories (≥ 500 kg, 350–500 kg and ≤ 350 kg).

According to the therapeutic trials which they received, cows were assigned into two groups: Group I (n = 60) received 100–150 ml intrauterine infusion of 0.5% Betadine solution (povidone-iodine, Nile Co. for Pharmaceutical and Chemical Industries, Cairo, Egypt) and Group II (n = 52) received 100–150 ml of 0.1% Septocid solution (dichloroxylenol, Chemical Industries Development, Giza, Egypt). Both treatments were repeated for at least two times at 7-day intervals. The treated cows were examined gynaecologically every week thereafter to monitor the results of treatment. The responding cows exhibiting an apparently normal uterus and cervix with cessation of the abnormal vaginal discharge were inseminated 10–12 h after oestrus detection using frozen semen from bulls of proven fertility. The success or failure of the treatment was evaluated on the basis of post-treatment conception rates two months later.

Statistical differences in recovery and conception rates were tested by Chi-square analysis as described by Snedecor and Cochran (1967).

Results

On rectal examination, the ovaries of repeat-breeder cows revealed normal size and texture. They were normally cycling as there was either a Graafian follicle or a corpus luteum at different stages of growth. The uteri showed a varying degree of enlargement, hardness and thickness, and so did the cervix. Vaginal examination showed hyperaemia of the cervix in the cranial part of the vagina, accompanied with a small amount of abnormal discharge.

The effect of Betadine and Septocid treatment on endometritis and cervicitis in dairy cows is shown in Table 1. The percentage of animals responding to an intrauterine infusion of Betadine and their conception rate (78.3% and 61.7%, respectively) were found to be significantly higher ($P < 0.01$) than those of cows infused with Septocid (30.8% and 9.6%, respectively). The relationship between the body weight of cows and the results of treatment with Betadine and Septocid are shown in Table 2. The recovery and the conception rates were generally higher as the body weight increased. Table 3 represents the relationship between the length of open days prior to treatment and the results of treatment with Betadine and Septocid in cows. When the pretreatment open days decreased, the response of animals to treatment as well as the conception rate increased. The higher number of previous services hindered the response of cows to treatment (Table 4).

Table 1

Influence of Betadine and Septocid on endometritis and cervicitis in cows

Type of affection	Betadine			Septocid		
	Number of cows	Recovery rate	Conception rate	Number of cows	Recovery rate	Conception rate
Endometritis	20	80.0% (16)	60.0% (12)	25	32.0% (8)	8.0% (2)
Cervicitis	25	88.0% (22)	76.0% (19)	22	31.8% (7)	13.6% (3)
Endometritis and cervicitis	15	60.0% (9)	40.0% (6)	5	20.0% (1)	0.0% (0)
Total	60	78.3% (47)	61.7% (37)	52	30.8% (16)	9.6% (5)

Figures in parentheses represent the number of cows

Table 2

Relationship between the body weight of cows and the results of treatment with Betadine and Septocid

Body weight	Betadine									Septocid								
	Endometritis			Cervicitis			Endometritis and cervicitis			Endometritis			Cervicitis			Endometritis and cervicitis		
kg	n	RR	CR	n	RR	CR	n	RR	CR	n	RR	CR	n	RR	CR	n	RR	CR
≥ 500	10	90.0% (9)	70.0% (7)	13	92.3% (12)	84.6% (11)	8	50.0% (4)	37.5% (3)	11	36.4% (4)	9.1% (1)	9	33.3% (3)	22.2% (2)	1	0.0% (0)	0.0% (0)
350–500	6	83.3% (5)	66.4% (4)	7	85.7% (6)	71.4% (5)	4	75.0% (3)	75.0% (3)	8	37.5% (3)	12.5% (1)	11	27.3% (3)	0.0% (0)	2	50.0% (1)	0.0% (0)
≤ 350	4	50.0% (2)	25.0% (1)	5	80.1% (4)	66.0% (3)	3	66.7% (2)	0.0% (0)	6	16.7% (1)	0.0% (0)	2	50.0% (1)	50.0% (1)	2	0.0% (0)	0.0% (0)
Total	20	80.0% (16)	60.0% (12)	25	88.0% (22)	76.0% (19)	15	60.0% (9)	40.0% (6)	25	32.0% (8)	8.0% (2)	22	31.8% (7)	13.6% (3)	5	20.0% (1)	0.0% (0)

n = number of cows; RR = recovery rate; CR = conception rate. Figures in parentheses represent the number of cows

Table 3

Relationship between the length of service period (open days) and the results of treatment with Betadine and Septocid in cows

Open days	Betadine									Septocid								
	Endometritis			Cervicitis			Endometritis and cervicitis			Endometritis			Cervicitis			Endometritis and cervicitis		
	n	RR	CR	n	RR	CR	n	RR	CR	n	RR	CR	n	RR	CR	n	RR	CR
< 60–120	3	100% (3)	100% (3)	6	83.3% (5)	83.3% (5)	4	100% (4)	75.0% (3)	9	44.4% (4)	11.1% (1)	7	42.9% (3)	14.3% (1)	3	33.3% (1)	0.0% (0)
121–180	8	87.5% (7)	75.0% (6)	10	100% (10)	90.0% (9)	3	66.7% (2)	66.7% (2)	6	33.3% (2)	16.7% (1)	5	20.0% (1)	0.0% (0)	1	0.0% (0)	0.0% (0)
181–240	6	66.7% (4)	33.3% (2)	5	80.0% (4)	80.0% (4)	4	75.0% (3)	25.0% (1)	6	16.7% (1)	0.0% (0)	4	25.0% (1)	25.0% (1)	0	0.0% (0)	0.0% (0)
> 241	3	66.7% (2)	33.3% (1)	4	75.0% (3)	25.0% (1)	4	0.0% (0)	0.0% (0)	4	25.0% (1)	0.0% (0)	6	33.3% (2)	16.7% (1)	1	0.0% (0)	0.0% (0)
Total	20	80.0% (16)	60.0% (12)	25	88.0% (22)	76.0% (19)	15	60.0% (9)	40.0% (6)	25	32.0% (8)	8.0% (2)	22	31.8% (7)	13.6% (3)	5	20.0% (1)	0.0% (0)

n = number of cows; RR = recovery rate; CR = conception rate. Figures in parentheses represent the number of cows

Table 4

Relationship between the number of previous services and the results of treatment with Betadine and Septocid in cows

Number of previous services	Betadine									Septocid								
	Endometritis			Cervicitis			Endometritis and cervicitis			Endometritis			Cervicitis			Endometritis and cervicitis		
	n	RR	CR	n	RR	CR	n	RR	CR	n	RR	CR	n	RR	CR	n	RR	CR
2–4	7	100% (7)	85.7% (6)	9	100% (9)	100% (9)	6	83.3% (5)	66.7% (4)	11	45.5% (5)	9.1% (1)	10	40.0% (4)	20.0% (2)	1	100% (1)	0.0% (0)
5–7	4	100% (4)	75.0% (3)	7	85.7% (6)	85.7% (6)	3	66.7% (2)	33.3% (1)	7	28.6% (2)	0.0% (0)	5	20.0% (1)	20.0% (1)	2	0.0% (0)	0.0% (0)
8–10	3	66.7% (2)	33.3% (1)	4	75.0% (3)	50.0% (2)	5	40.0% (2)	20.0% (1)	5	20.0% (1)	20.0% (1)	3	33.3% (1)	0.0% (0)	0	0.0% (0)	0.0% (0)
> 10	6	50.0% (3)	16.7% (2)	5	80.0% (4)	40.0% (2)	1	0.0% (0)	0.0% (0)	2	0.0% (1)	0.0% (0)	4	25.0% (1)	0.0% (0)	2	0.0% (0)	0.0% (0)
Total	20	80.0% (16)	60.0% (12)	25	88.0% (22)	76.0% (19)	15	60.0% (9)	40.0% (6)	25	32.0% (8)	8.0% (2)	22	31.8% (7)	13.6% (3)	5	20.0% (1)	0.0% (0)

n = number of cows; RR = recovery rate; CR = conception rate. Figures in parentheses represent the number of cows

Discussion

Endometritis and cervicitis represent about 60–78.0% of infertility and sterility problems in cows (Langer, 1958; Schmidt, 1971).

Treatment of endometritis and cervicitis in cows is based on either promoting the oestrous cycle (Heim, 1954) and/or overcoming the suspected or known intrauterine infection (Ohm, 1955). Intrauterine infusions with antibiotics (Khan et al., 1991) or chemical disinfectants (Pundt, 1950; Butayeva, 1958) have been tried out. Although both approaches showed a varying degree of success, chemical disinfectants are superior as they leave no residues in the body tissues of treated cows as antibiotics do (Brander et al., 1991). Iodine is rapidly absorbed from the uterus. Its beneficial action may be due to its antimicrobial activity and to the local irritation, leucocytosis and hyperaemia it produces, and it may correct an iodine deficiency (Moberg, 1961). Recently, douching the uterus with chlorhexidine or Betadine in 2% stock solution (due its povidone iodine content) has been shown to have a mild irritating action on the endometrium, resulting in the mobilization of leukocytes and stimulation of uterine motility, and it has antiseptic and flushing actions on any mucopurulent material (Roberts, 1986). Moreover, non-iodinic antiseptics such as Septocid (dichloroxylenol) solution are widely used for irrigation of the uterus and vagina in a concentration of 1–2% (Brander et al., 1991).

Our results revealed a recovery rate of 78.3% as well as a conception rate of 61.7% after the intrauterine administration of Betadine in cows suffering from endometritis and/or cervicitis. This recovery rate was in agreement with the previous findings obtained after Lugol's iodine by Gupta et al. (1989) (74.8%), Lotagen by Blicharski (1962), Schmidt (1971) and Zhang and Chen (1989) (59.6%–95.0%), and iodine treatment by Schnellbach (1990) (71.0%). The previously reported conception rates obtained after treatment with Lugol's iodine (Gupta et al., 1989) and iodine (Münker, 1955) are consistent with those found in the present study, and ranged between 62 and 72%. Betadine (due to its povidone content) is less irritating and is more rapidly absorbed than Lugol's iodine. Besides its antiseptic action, it also stimulates uterine motility. Betadine will cause flushing of any mucopurulent material, which facilitates the elimination of the causative agents of endometritis (Roberts, 1986).

Septocid, as a solution of 1–2% concentration, is widely used for irrigation of the uterus and vagina (Brander et al., 1991), but the recovery (30.8%) as well as the conception (9.6%) rates of treated cows were not parallel with those obtained after Betadine (Table 1). Thus it could be concluded that iodine preparations, especially those containing povidone (Betadine), are more efficient in the control of genital infection in cows than other chemical disinfectants.

Table 2 shows the better uterine recovery and the relatively high conception rate in group I (cows weighing ≥ 500 kg) as compared to the other groups. This may be due to the fact that the uterus of the former cows has built a better defence

mechanism which helps rapid endometrial healing (Roberts, 1986) and, consequently, a higher conception rate.

It was also proved that cows inseminated between post-partum day 60 and 180 (Table 3) and having 4–7 inseminations (Table 4) respond better to treatment than do other cows. This is not surprising, as these cows were normally cycling and their genitalia were under the influence of oestrogen (for several cycles) which has a well-known flushing and antimicrobial action (Butayeva, 1958; Sharaf et al., 1963). This is supported by the early statement of Miller and Ras (1952) that a deficiency of hormone production, especially for cleaning at the time of oestrus, will predispose the uterus to infection.

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COMPARATIVE CLINICAL AND BIOCHEMICAL ANALYSES OF TWO METHODS OF PANCREATIC CANNULATION IN PIGS

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The authors' method of long-lasting pancreatic cannulation from the side of the duodenal papilla was compared with the method of accessory pancreatic duct direct cannulation in 20 pigs divided into two groups of 10 animals each. The volume of pancreatic juice was determined in both groups. In addition, the parameters of acid-base equilibrium in whole blood and the values of Na^+, K^+, Cl^-, pH and HCO_3^- in the pancreatic juice were determined. More efficient drainage was obtained with the authors' method. However, due to the longer collection of pancreatic juice multielectrolytic compensation liquids had to be used in the animals to maintain acid-base equilibrium and to prevent the risk of metabolic acidosis.

Key words: Pancreas, cannulation, pigs, pancreatic juice

The functioning of the pancreas in physiological and pathological conditions has been the subject of numerous studies. Due to the large number and high complexity of pancreatic functions comprising exocrine and endocrine activities, all pathological conditions of the pancreas are of particularly great importance.

Many laboratory techniques have been developed for indirect examination of secretory functions of the pancreas by determining the activity of its enzymes in blood, urine and faeces, along with so-called functional tests and radiological examinations, both classical ones and those using contrast media, ultrasonography, computer tomography and many others (Bożkowa, 1961; Konturek, 1976; Piotrowski et al., 1984; Złamaniec and Bryc, 1985; Konturek, 1987; Łukasiewicz and Zaik, 1988).

The possibility of obtaining pancreatic juice over long periods of time constitutes a particulary valuable research tool in medicine and experimental veterinary medicine. Various methods of long-lasting pancreatic cannulation create such a possibility. Research conducted in this direction has resulted in the elaboration of a number of operational techniques in different species of animals. Although there are a lot of methods for long-lasting pancreatic cannulation, none of them is perfect.

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The aim of this study was to perform a comparative evaluation of the authors' method of long-lasting pancreatic cannulation from the side of the duodenal papilla vs. the method of accessory pancreatic duct direct cannulation.

Materials and methods

Twenty pigs of both sexes, weighing approximately 80–90 kg, were used. Two methods of long-lasting pancreatic cannulation were employed, and the experimental animals were divided into two groups (I and II) of 10 pigs each.

Atropine 0.04 mg/kg s.c. and propionylpromazine (Combelen, Bayer) 0.4 mg/kg i.m. were used for premedication of pigs, and pentobarbital (Vetbutal, Biowet) 15–20 mg/kg i.v. was applied for general anaesthesia.

The method of accessory pancreatic duct direct cannulation was used on pigs of group I (Konturek et al., 1970; Klein et al., 1981; Hee et al., 1988; Matsuno et al., 1991). After opening abdominal integuments in the last intercostal space on the right side, the cephalad section of the duodenum was exteriorized. Subsequently the accessory pancreatic duct was located, incised transversely, and a previously prepared polyethylene cannula provided with 3 cuffs was introduced into its lumen. The first of the cuffs served as a support for the binder, fastening the cannula in the accessory pancreatic duct, while the remaining two were a support for the seam, fastening the cannula at the duodenal wall. The ending part of the cannula was led out of the abdominal integuments (Figs 1 and 3).

In group II, the authors' method of long-lasting pancreatic cannulation was used. Abdominal integuments were also opened in the last intercostal space on the right side. After exteriorizing the proximal section of the duodenum and locating the accessory pancreatic duct, the duodenum was incised above the minor duodenal papilla. A previously prepared cannula, made of polyethylene and provided with a transverse arm fastened between two cuffs, was introduced into the exposed papilla. After introducing the cannula and sealing up with a binder the accessory pancreatic duct around it, the duodenal incision was closed in such a way that the transverse arm remained in its lumen, preventing the cannula from slipping out (Figs 2 and 4).

In both groups, the cannulas had a similarly shaped ending: slanting, provided with 5 side holes, with a diameter approximating its lumen. The cannulas were led out to the body surface in the upper part of the wound of the abdominal integuments, and directly into plastic, flat containers fastened onto the body surface, where pancreatic juice was collected. The period of observation was 14 days in both groups.

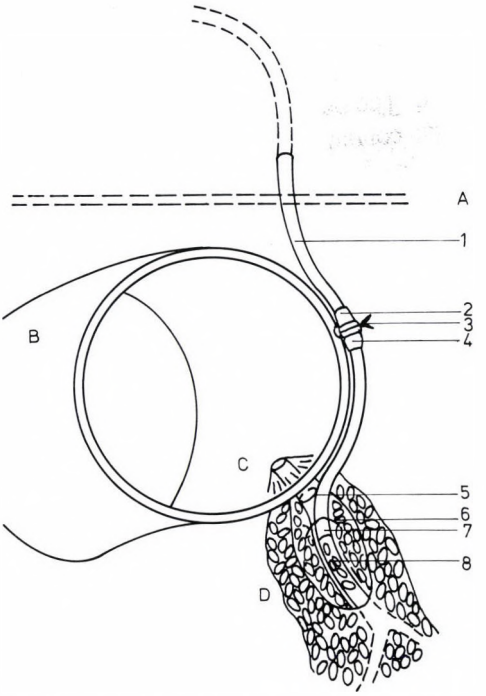


Fig. 1. Method of accessory pancreatic duct direct cannulation used in group I. A: abdominal wall; B: duodenum; C: minor duodenal papilla; D: pancreas; 1: cannula; 2: polyethylene cuff; 3: ligature; 4: polyethylene cuff; 5: ligature; 6: ligature; 7: polyethylene cuff; 8: end of cannula

Besides, a determination of acid-base equilibrium (ABE) parameters in whole blood and of Na^+, K^+, Cl^-, pH and HCO_3^- levels in the pancreatic juice was done in pigs of group II 1, 3 and 6 days after the procedure. The clinical symptoms of metabolic acidosis, observed in pigs of group I prompted us to study, in animals of group II, the acid-base balance of the blood and the level of electrolytes and bicarbonates in the pancreatic juice, which we originally had not intended to include in the experiment. This is why these parameters were not determined for group I. The parameters of ABE were analysed by Astrup's method, while the level of sodium and potassium in the pancreatic juice was determined with the Easy-Lite analyser and that of HCO_3^- was measured with Plastomed 206. The results of biochemical tests were expressed in SI units and subjected to statistical evaluation by Student's *t*-test.

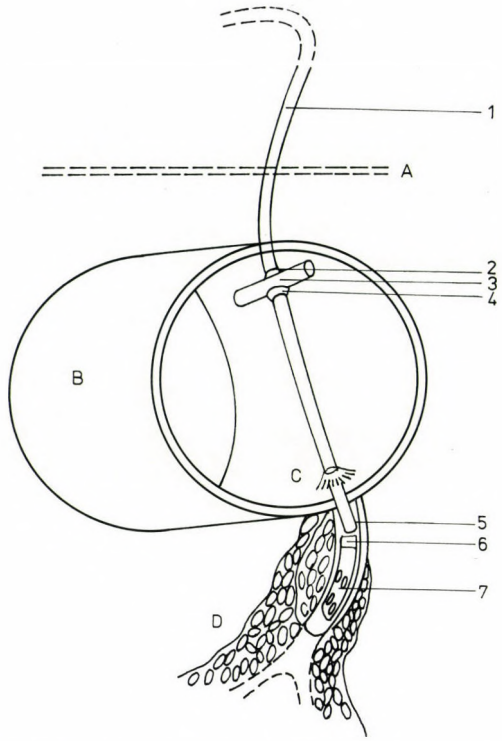


Fig. 2. The authors' method of pancreatic cannulation used in group II. A: abdominal wall; B: duodenum; C: minor duodenal papilla; D: pancreas; 1: cannula; 2: polyethylene cuff; 3: transversal arm; 4: polyethylene cuff; 5: ligature; 6: ligature; 7: polyethylene cuff; 8: end of cannula

Results and discussion

No complications occurred in the pigs of either experimental group after surgery. The volume of pancreatic juice obtained with the cannulas was 39, 413 and 243 ml in group I and 125, 588 and 425 ml in group II 1, 3 and 6 days after the procedure, respectively (Table 1).

The volumes of pancreatic juice obtained from pigs of group II were considerably higher than those collected from animals of group I, indicating that the authors' method of long-lasting pancreatic cannulation enabled a more efficient drainage.

Between days 1 and 3 after surgery, the health status of group I animals did not change. Body temperature remained within the physiological limits and the animals' appetite and thirst were not affected. Three days after the operation, the

first symptoms of acidosis appeared in pigs of group I. At that time, decreased appetite, depression, tachypnoea, and hyperaemia of the mucous membranes were observed. The clinical symptoms observed in pigs of that group prompted us to analyse the acid-base equilibrium of the blood and determine the levels of electrolytes and bicarbonates in the pancreatic juice of animals of group II.



Fig. 3. Method of accessory pancreatic duct direct cannulation used in group I

The analysis of ABE indicators showed that metabolic acidosis developed between days 3 and 6 of the experiment (Table 1). The blood pH tended to be more acidic (7.31–7.28), with a simultaneous fall in the HCO_3^- content (21.4–19.9 mmol/l) and a decrease of BE (–8.0 mmol/l, –12.6 mmol/l). The values of pCO_2 rose 3 days after the operation (8.25 kPa), indicating a compensated metabolic acidosis, while after the 6th day they fell (4.52 kPa) as a sign of non-compensated metabolic acidosis.

The pancreatic juice had an alkaline pH which increased during the experiment (day 1: 8.01, day 3: 8.12, day 6: 8.28). The level of bicarbonates, the basic anions of the pancreatic juice, increased in proportion to the volume of juice obtained, and amounted to 68.0 mmol/l (day 1), 127.0 mmol/l (day 3), and 158.0 mmol/l (day 6).



Fig. 4. The authors' method of pancreatic cannulation used in group II

Table 1

Volume of pancreatic juice obtained and ABE indicators in the whole blood of experimental pigs

Indicators	Duration of experiment					
	1 day		3 days		6 days	
	Group I	Group II	Group I	Group II	Group I	Group II
Volume of pancreatic juice, ml	39.0	125.0*	413.0**	588.0**	243.0*	425.0**
Acid-base equilibrium (ABE) indicators						
pH	–	7.38**	–	7.31*	–	7.28
pCO^2, kPa	–	6.44*	–	8.25**	–	
HCO_3^-, mmol/l	–	26.7**	–	21.4*	–	18.9
BE, mmol/l	–	(–)2.0	–	(–)8.0*	–	(–)12.6**

*P = 0.05 (estimated); **P = 0.01 (estimated)

The chloride concentration of the pancreatic juice was inversely proportional to the concentration of bicarbonates (110.0 mmol/l, 48.0 mmol/l and 19.0 mmol/l (on days 1, 3 and 6, respectively). The basic cations of the pancreatic juice, sodium and potassium, remained on a level close to their concentrations in the serum, and their concentration did not depend either on the duration of the experiment or on the volume of pancreatic juice obtained (Table 2).

Table 2

Average values of pH and levels of HCO_3^-, Na^+, K^- and Cl^- in pancreatic juice from pigs of experimental group II

Indicators	Duration of experiment		
	1 day	3 days	6 days
pH	8.01	8.12	8.28
HCO_3^-, mmol/l	68.0	127.0*	158.0**
Na^+, mmol/l	142.4	148.3	147.6
K^+, mmol/l	5.8	5.6	5.9
Cl^-, mmol/l	110.0**	48.0*	19.0

*P = 0.05 (estimated); **P = 0.01 (estimated)

In pigs of group II, clinical symptoms similar to, but usually more severe than those seen in group I were observed. The animals had a decreased appetite and excreted a markedly dehydrated faeces. The clinical symptoms seen after pancreatic cannulation were obviously correlated with the volume of pancreatic juice obtained.

With many methods of obtaining pancreatic juice from experimental animals, the juice is collected together with pancreatic secretion, which excludes many precise biochemical examinations. This shortcoming is not restricted to the oldest methods (Pawlow, 1910; Inlow, 1921; Babkin, 1928). Similar defects appear in modern methods which create a closed section of pancreas together with opening the pancreatic duct and connecting it with the cannula and the body integument (Konturek et al., 1976; Chey and Konturek, 1982; Chen et al., 1983; Zebrowska et al., 1983; Hee et al., 1985; Sakamoto et al., 1988). These are very laborious methods which underestimate the work of the alimentary tract. The methods suggested by Thomas and Crider (1940), Konturek et al. (1970), Klein et al. (1981) and Matsuno et al. (1991) require an immobilization of the duodenum at the wall of abdominal cavity and obtain pancreatic juice through a cannula which is permanently introduced into the pancreatic duct or introduced under control for the period of the experiment. The pancreatic juice thus obtained is non-active and free from other secretions. The disadvantage of this method is that the

pancreas is immobilized, which changes the conditions of alimentary tract functioning and causes stress to the animals each time when the cannula is introduced into the pancreatic duct.

The method which is nearly ideal is based on an introduction of the cannula into the pancreatic duct next to the opening of the pancreatic duct into the duodenum (Nunes and Corring, 1979; Partridge et al., 1982; Ormai et al., 1986; Kato et al., 1987; Konturek, 1987; Pierzynowski et al., 1988). In these methods interference with the alimentary tract is limited to the minimum and the sampling of pancreatic juice causes very little stress to the animals. However, in pigs the pancreatic duct is in most cases covered by pancreatic parenchyma, which enables the introduction of the cannula without injuring the pancreas. This has prompted the authors to develop a new method by which clean, non-active pancreatic juice can be obtained without injuring the anatomic structures and causing very little stress to the experimental animals.

It is noteworthy that the method developed for obtaining pancreatic juice by cannulation of the pancreatic duct in pigs yields pancreatic juice in a volume sufficient for laboratory analysis. However, because of the risk of metabolic acidosis, during prolonged collection multielectrolytic compensatory liquids should be given to the animals to maintain the acid-base equilibrium.

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COMING EVENTS

XIth Congress of the World Veterinary Poultry Association (WVPA)

18–22 August 1997

Budapest
HUNGARY

INVITATION

On behalf of the Organizing Committee, we take great pleasure in inviting you to participate in the XIth WVPA Congress. This is the first time that the Congress will have an East/Central European venue and we hope that you will be able to enjoy many of the interesting cultural and scenic aspects of our beautiful country.

We are planning a conference which will have a broad range of topics. It will include papers on scientific research as well as day to day practice. You can assist us in this process by returning the reply card indicating your particular areas of interest or possible input. We should be grateful if this could be sent as soon as reasonably possible.

In conjunction with the Congress we will arrange an enjoyable social programme which will provide an opportunity to meet and interact with other participants. There will be trips available to places of interest before and after the conference. Full information will be made available in good time.

We hope to renew many friendships from previous conferences and make new ones in 1997.

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Congress Organizing Committee

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Head
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and Food Inspection
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THE SCIENTIFIC PROGRAMME

Papers will be given covering the topics listed below, as well as other topics suggested by input from participants. One of the major objectives of this Congress will be to **"Bridge the Gaps"**. This means addressing the problems associated with the transfer of knowledge from the molecular biology research laboratory to the producer and the professional in the field. A second "gap" is the one between the technology and knowledge of the developed countries and that of areas which are struggling. The organizers will have this focus in mind when producing the final programme of scientific sessions and workshops.

The organizers will call for oral or poster presentations on topics as listed below:

1. Structure and function of the avian immune system
2. Viral, bacterial, mycoplasmal and parasitic diseases of poultry
3. Epidemiology and diagnosis of avian diseases in the age of molecular biology
4. Developments in vaccine research
5. Drugs and therapeutics for poultry
6. Poultry and food safety
7. Role of genetics in disease prevention
8. Poultry waste management, disinfection and disease control
9. Developmental, nutritional and metabolic diseases
10. Computers in poultry health and management
11. Trends in poultry research and education of poultry veterinarians
12. Role of biosecurity and management in disease control including welfare aspects

Keynote speakers will be invited to make presentations on selected topics.

DATES AND VENUE

The XIth WVPA International Congress will take place from 18 to 22 August 1997 at the Budapest Convention Centre.

This venue is centrally and attractively located and is easily reached by public transport from all major hotels.

SOCIAL AND CULTURAL ACTIVITIES

The social programme includes a welcoming party, a farewell party and sight-seeing tours.

Hungary is well known for its hospitality, and Budapest with its marvellous location is considered by many as one of the most beautiful cities of the Danube river countries. The weather in Budapest in August is warm with a late summer temperature of 25 °C, accompanied by occasional light showers. The opportunity will be offered to become acquainted with real "puszta" life and the landscape of Hortobágy National Park, Bábolna, one of the biggest poultry breeding centres in Hungary, and other scenic spots by way of guided tours and excursions.

Participants, and those accompanying them, will be welcome at all events.



The 22nd Congress of the German Veterinary Medical Society will be held from 8 to 11 April 1997 in Bad Nauheim (near Frankfurt/Main), Germany. The topic is

"Current Research Results in Veterinary and Comparative Medicine".

Papers and posters can be presented in German or English. The title of only unpublished papers (10 min) and posters should be submitted until **31 August 1996** to the President of DVG, Prof. Dr. Dr.h.c. mult. E. Grunert, Klinik für Geburtshilfe, Bischofsholer Damm 15, D-30173 Hannover/Germany;
Phone ++49–511–856–7242, Fax ++49–511–856–7691.

Der 22. Kongreß der Deutschen Veterinärmedizinischen Gesellschaft findet vom 8. bis 11. April 1997 in Bad Nauheim statt. Zum Themenbereich

"Aktuelle Forschungsergebnisse aus der Veterinärmedizin und vergleichenden Medizin"

können ab sofort Kurzvorträge und Posterbeiträge mit neuen, noch nicht vorgetragenen bzw. publizierten Ergebnissen angemeldet werden. Anmeldeschluß für Vorträge und Poster ist der **31. August 1996.**
Anmeldungen an den Vorsitzenden der DVG Prof. Dr. Dr.h.c. mult. E. Grunert, Klinik für Geburtshilfe, Bischofsholer Damm 15, 30173 Hannover, Deutschland;
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NOTES FOR CONTRIBUTORS

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Papers submitted for publication are subject to peer review. Acceptance depends on the opinion of referees and the decision of the Editorial Board. Papers accepted for publication are subject to editorial revision. Only original papers will be published and a copy of the Publishing Agreement will be sent to the authors of papers accepted for publication. Manuscripts will be processed only after receiving the signed copy of the agreement.

MANUSCRIPT STYLE

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

FORM OF MANUSCRIPT

Title. The title should be a clear and concise statement of the contents in not more than 14 words. A short running title of not more than 50 characters should also be supplied. This is followed by the authors' initials (full first name of women) and surname, and the name of the institution where the work was done. The mailing address of the authors must also be indicated here.

Abstract. This should not exceed 200 words and should outline briefly the purpose of the study and detail important findings and the authors' principal conclusions. Redundant phrases, generally known information and repetition should be avoided. **Key words** (4–6) should also be supplied.

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

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Acknowledgement of grants and technical help.

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STUDIES ON THE GROWTH OF RABBITS BY X-RAY COMPUTERISED TOMOGRAPHY

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(Received March 25, 1996; accepted May 28, 1996)

X-ray computerised tomography (CT) was used for determining changes in the tissues of growing rabbits. Serial scans were taken on Pannon White rabbits weighing of 0.5, 1.5, 2.5 and 3.5 kg (5 animals per group). Twenty-four scans per animal were evaluated, from the scapular arch to the end of the femur. Three-dimensional (3D) histograms were constructed between –200 and +200 HU value, which represented the serial number of scans (24 scans) on the X axis, the density of the picture-forming pixels (in HU variables) on the Y axis, and the frequency of density values on the Z axis. The 3D histograms show the tissue composition of rabbit bodies, with different peaks corresponding to the scapular arch, the m. longissimus dorsi and the hindleg muscles. Fat depots were also definitely demonstrated, with different peaks in the scapular, abdominal and pelvic regions. The volume and the anatomical location of fat and muscle tissue were determined. An abrupt increase in fat content was observed between 2.5 and 3.5 kg of body weight.

Key words: Rabbit, computerised tomography, growth, body composition

X-ray computerised tomography (CT) is a powerful tool for *in vivo* studies on the body composition of rabbits. The theoretical basis of the method is that different types of tissues absorb X-rays in dissimilar degrees. On a scale named after Houndsfield these tissues can be separated on the basis of their density values. At the beginning of the 1980s, Norwegian researchers (Skjervold et al., 1981) proved that the tissues of animals can be characterised by non-overlapping scale intervals.

At the Pannon University of Agricultural Sciences, a Siemens Somatom DRG type, third-generation CT scanner started working in 1990 (Horn, 1991). At the end of 1994 a more advanced, so-called spiral CT was installed in the CT Biological Centre of the university. At present the two instruments are in use, together with a magnetic resonance imaging (MRI) apparatus, for both medical and animal science research.

The use of CT scanning in rabbit research was started in 1991. In the first experiments the carcass traits of meat-type rabbits were estimated. The L value,

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i.e. the average surface of the m. longissimus dorsi (mLD) between the 2nd and 3rd, and the 4th and 5th lumbar vertebrae, was found to be a good indicator of dressing percentage (Szendrő et al., 1992). Since then this value has been used in the breeding programme of Pannon White rabbits. From subsequent experiments it was concluded that selection of growing rabbits for the L value is an effective method for increasing the dressing percentage (Szendrő et al., 1994; Romvári et al., 1995). Fekete et al. (1993) studied the nutrient requirement of gestation in rabbits by CT and by direct chemical analysis carried out at different stages of pregnancy. Changes occurring in the body composition of rabbits during pregnancy were determined by Milisits et al. (1996).

Romvári et al. (1996) summarised the essential features of the imaging and scanning procedure and the possibilities of picture evaluation.

The growth of rabbits and the quantitative and qualitative changes in their body composition have been in the focus of investigations for a long time. Vezinhet and Prud'Hon (1975), Ouhayoun (1983), and Deltoro and Lopez (1985) analysed the allometric growth of different tissues and organs. The carcass traits have been analysed by Varewyck and Bouquet (1982), Petersen et al. (1988), Parigi-Bini et al. (1992), and Szendrő et al. (1996). In the above-mentioned studies the lack of an *in vivo* method caused difficulties in checking the same animal repeatedly during its life.

The objective of this study was to develop a new *in vivo* method which is based on serial CT scans and provides possibility for measuring changes that occur in different tissues of rabbits during growth.

Materials and methods

The study was carried out at the Faculty of Animal Sciences of the Pannon University of Agricultural Sciences in Kaposvár. Twenty young Pannon White rabbits were examined in four weight categories (0.5, 1.5, 2.5 and 3.5 kg) using 5 rabbits in each group. The animals were scanned at night after six hours' starvation. During the examination the rabbits were fixed with belts in outstretched position, lying flat in a special "container" without anaesthesia.

The body of rabbits was scanned from the scapular arch to the end of the femur. First the length of the vertebral column was measured from the 5th cervical vertebra to the head of the femur. In each weight category this was divided by 20 to get the distance between the two neighbouring scans. As a result, scans of the same serial number belong to the same anatomical sites in animals of different size, thus allowing their comparison. The pictures were taken from the direction of the head. After reaching the head of the femur (20th scan), some additional scans

were taken, down to the femorotibial articulation. Finally a total of 24 pictures were evaluated from each animal.

On the right side of Fig. 1 there is a side-taken topogram, where the anatomical location of the picture (centre) and the borders (double lines) of the scanned body interval can be seen. The histogram on the left was constructed from the pixel density values of the marked area. It has two peaks: one for the fat and one for the muscle tissues.

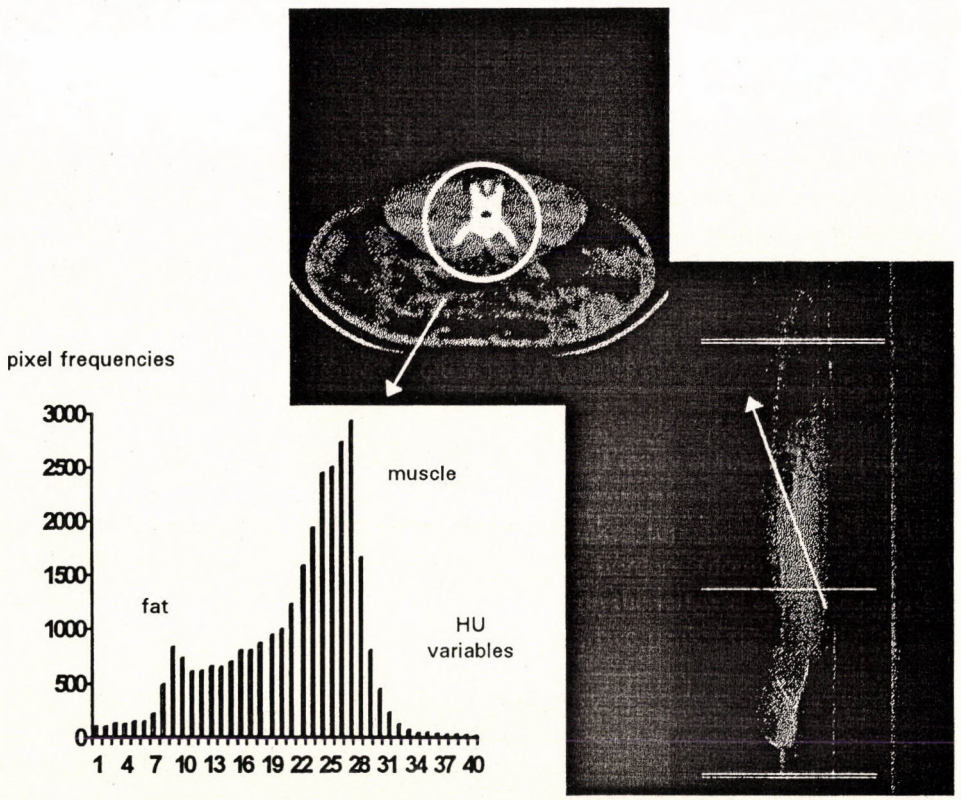


Fig. 1. Construction of a histogram (left) from the pixel density values of the marked area. The anatomical location of the cut (centre) is shown on the topogram taken from the side (right)

The CT picture is a 256 × 256-element matrix. Each individual point of that matrix represents a CT value expressed in Houndsfield units and is called pixel. The pictures were evaluated with the help of the CTPC software (Berényi and Kövér, 1991) capable of recording the density values. For this work only that part of the whole Houndsfield scale was needed which contains the absorption interval of both the fat and the muscle tissue. [The scale spreads from −1000 (no

absorption) to +1000 Houndsfield units (HU) (total absorption), were the 0 point is the density value of water]. For the evaluation, every 10 consecutive values of the HU were summarised, and thus 40 data were formed in the interval between –200 and +200. The histograms based upon the data of frequency distribution were obtained in this way.

Basic data evaluation was carried out using a special software developed by us. The three-dimensional (3D) histograms were constructed by the method of negative exponential interpolation of the SYSTAT 5.01 package.

Results

Figure 2 shows a 3D histogram constructed from the data of rabbits weighing 0.5 kg. The serial number of the pictures is shown on the X axis, the HU variables on the Y axis (numbered from 40 to 1 after reduction by 10 from +200 to –200) and the frequency of density values on the Z axis. At this live-weight only muscle tissue can be seen.

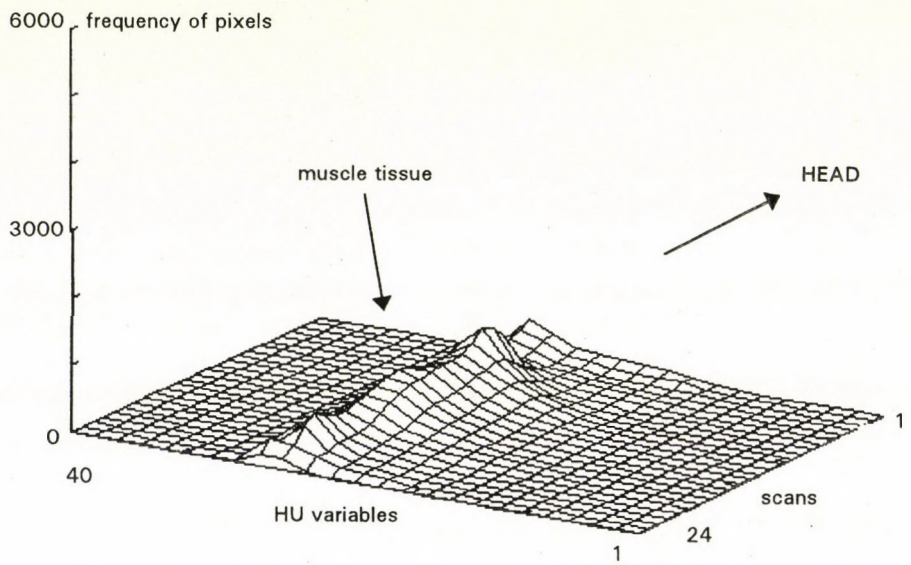


Fig. 2. Three-dimensional (3D) histogram of rabbits weighing 0.5 kg

In the case of animals weighing 1.5 kg, characteristic peaks are well separable on the mountain formed by the muscle tissue. Starting from the head, the first peak represents the periphery of the scapular arch. The following lower part is the interval of the lungs (4–5 scans), after which the biggest peak corresponding

to the mLD can be seen. The next lower part is the periphery of the pelvis (19th – 20th scan), which is distinct from the last peak formed by the thighs. The fat of the scapular arch and the perirenal fat appear on scans 1–3 and 12–14, respectively (Fig. 3).

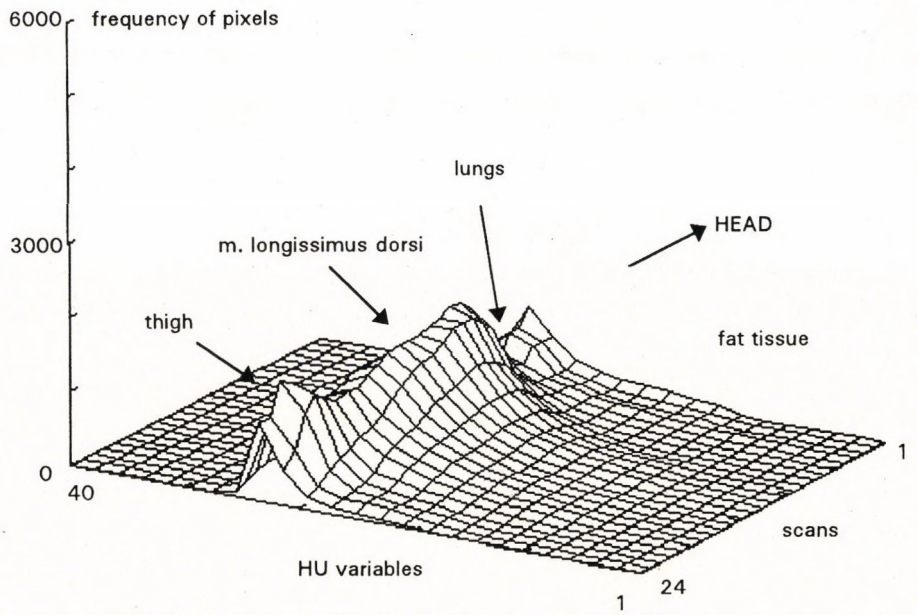


Fig. 3. Three-dimensional (3D) histogram of rabbits weighing 1.5 kg

Figure 4 shows the changes found in body composition between body weights of 0.5 and 1.5 kg, which is practically the difference of the two former 3D histograms. At this age (body weight) interval the whole trunk is quite smooth lengthwise and almost exclusively muscle tissue is being built in.

Figure 5 shows a histogram of rabbits weighing 2.5 kg. Apart of quantitative differences as compared to the previous histograms, this histogram shows the commencement of fat deposition around the pelvis (scans 19–21).

The last member of the series represents rabbits of 3.5 kg average body weight (Fig. 6). Compared with the previous histograms, here a significant increase can be seen in the amount of fat tissue. The fat depot around the kidney can be identified especially easily, and the fatness of the scapular arch is quite significant, too. The interval of the kidneys (scans 11–14) is enhanced in the figure. This is a really informative scan, because it cuts both the mLD and the region of renal fat. As mentioned above, there is a high correlation between the average surface of the mLD and the carcass traits. In the same way, the weight of renal fat is a good indicator of total body fat content.

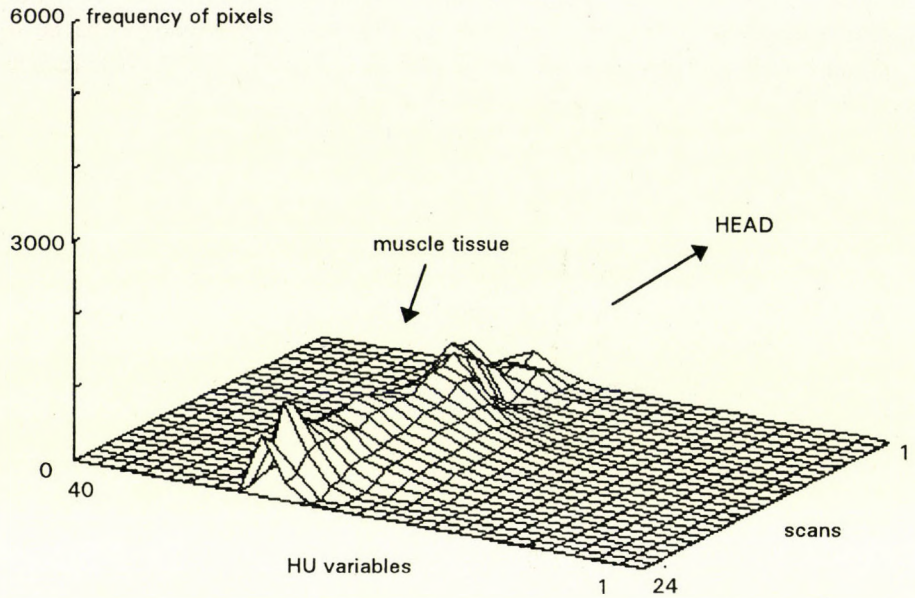


Fig. 4. Changes in body composition between body weights of 0.5 and 1.5 kg

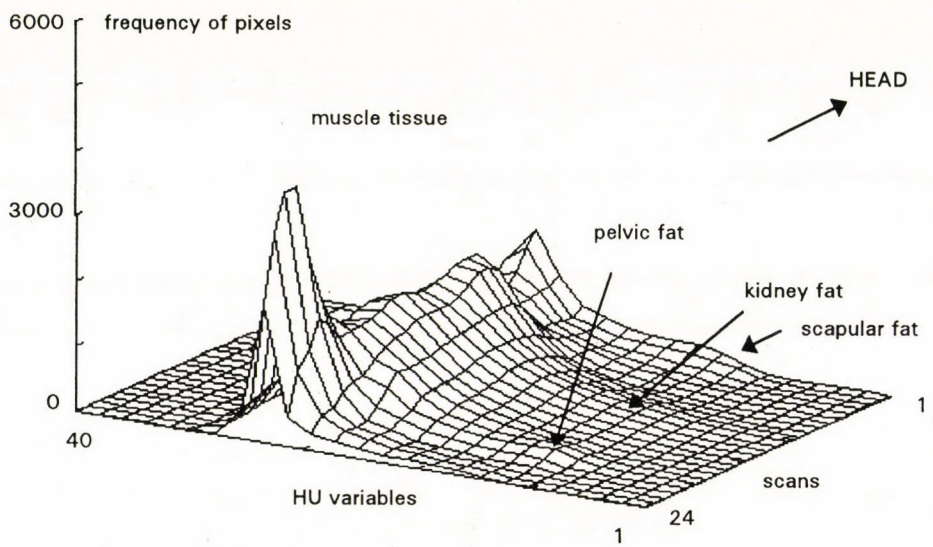


Fig. 5. Three-dimensional (3D) histogram of rabbits weighing 2.5 kg

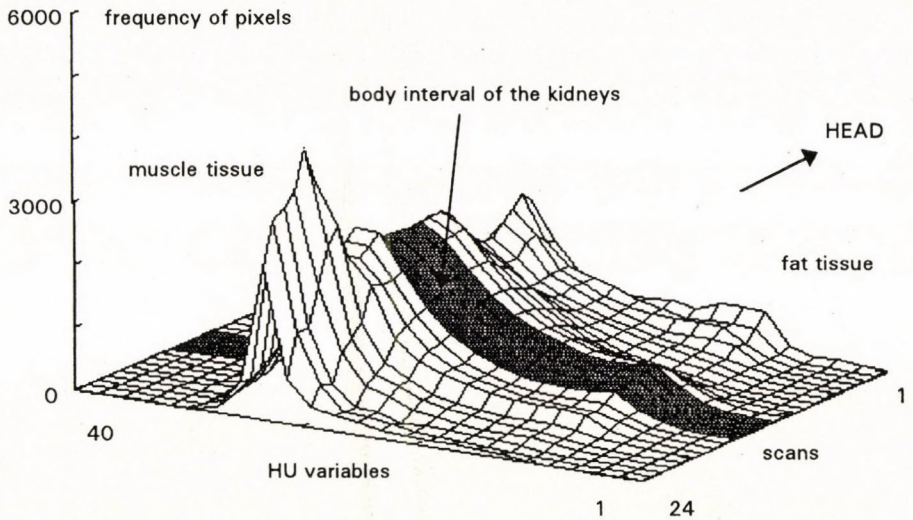


Fig. 6. Three-dimensional (3D) histogram of rabbits weighing 3.5 kg

Figure 7 demonstrates the changes occurring between body weights of 2.5 and 3.5 kg. As compared to the muscle tissue, the deposition of fat is surprisingly strong. This is characteristic of almost the whole body; however, it is the most significant around the kidneys and the scapular arch.

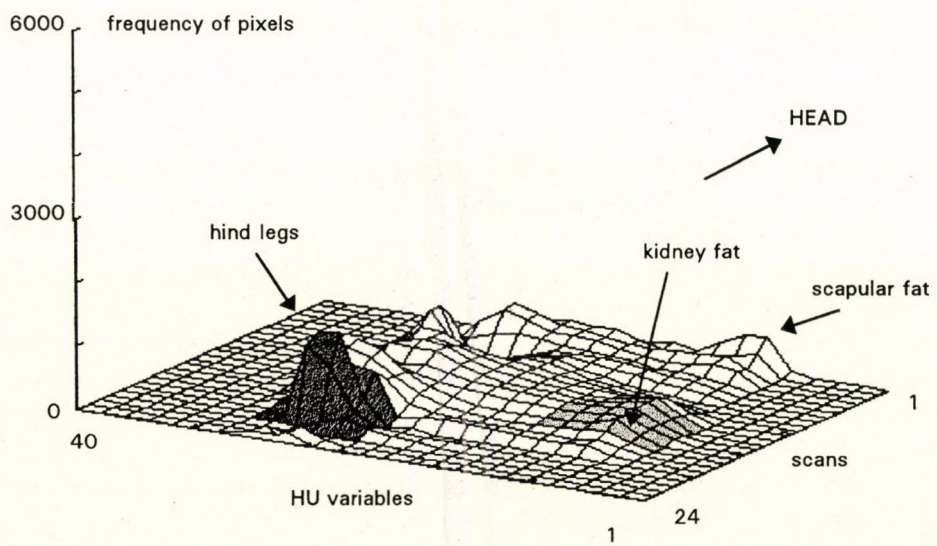


Fig. 7. Changes in body composition between body weights of 2.5 and 3.5 kg

Figure 8 shows the incorporation of fat and muscle tissue in the four weight categories examined within the interval of the kidneys (as marked in Fig. 6). The data were derived from the average values of 4 scans selected from each weight category. The histogram curve of rabbits weighing 0.5 kg has a characteristic peak within the interval of muscle. In the case of rabbits of 1.5 kg body weight, an increase can be seen in the weight of muscle tissue. The third curve belongs to rabbits of 2.5 kg body weight. It is at that stage that the fat tissue appears. The peak representing the muscle tissue shifts to the right, possibly because of the decrease in the water content of that tissue. Finally the last curve (representing rabbits of 3.5 kg body weight) has two characteristic peaks. Here, in addition to the increase of muscle tissue, an abrupt rise in fat content can be seen.

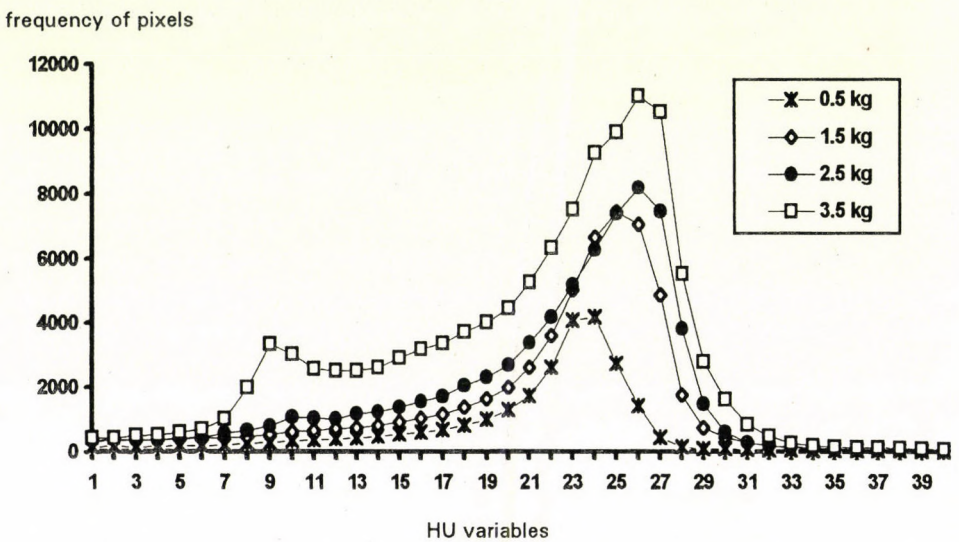


Fig. 8. Histograms of rabbits of the four weight categories between –200 and +200 Houndsfield intervals (HU variables 1–40)

CT pictures can be taken with an optional scan thickness (1–8 mm). This means that the picture-forming pixel can be considered a prism having a volume. Thus, it can be determined how big a part of its total volume falls within the HU interval relevant to the scan just examined. This enables the examiner to estimate the volume of e.g. fat tissue. By adding up data from several contiguous pictures, the volume of tissue within an optional body length can be determined. Total overlapping or, to characterise the distance between two pictures, the average value of two neighbouring scans can be used.

The data of 5 rabbits per group were processed as described above. Figure 9 shows the relative incorporation of both muscle and fat tissue in the renal region (scans 11–14). The base (100%) is the volume of these tissues in rabbits weighing 0.5 kg. The increase in the volume of muscle tissue was relatively uniform and continuous. The weight of muscle tissue was 3.5 times higher in rabbits weighing 3.5 kg than in rabbits of 0.5 kg body weight. As regards the fat tissue, an explicit breaking point could be demonstrated between the 2.5 kg and the 3.5 kg body weight categories: in rabbits weighing 3.5 kg an abrupt increase was demonstrable in the incorporation of fat tissue.

The fat content increased 8 times in the liveweight interval examined. It must be emphasized that this correlation is valid only between scans 11 and 14, and that the base value used for comparison was very low.

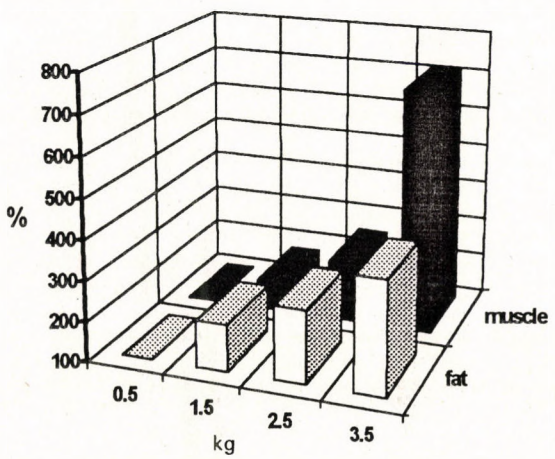


Fig. 9. Relative incorporation of tissues in the region of the kidneys in the four body weight categories

The changes observed in this study are consistent with the findings obtained by Vezinhet and Prud'Hon (1975), Ouhayoun (1983), and Deltoro and Lopez (1985) by experimental slaughtering. In harmony with their observations, the present study also revealed a drastic increase of the fat depots in rabbits weighing more than 2.5 kg, which increase is detrimental from the point of view of carcass quality. The method presented in this paper is suitable for predicting other carcass traits (e.g. the ratio of fore, intermediate and hind parts of the body or the valuable meat content of the carcass), too.

Discussion

The new *in vivo* method presented in this paper is suitable for analysing the growth of important tissues and predicting the body composition. By increasing the number of scans or decreasing the number of summarised X-ray density values, the efficiency of the method can be improved. As the animals can be tested repeatedly, the changes in their body composition can be followed throughout the growth period. The most important advantages of CT scanning are its non-invasive character and accuracy; however, the procedure is relatively expensive.

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MEASUREMENT OF THE TOTAL BODY FAT CONTENT OF GROWING RABBITS BY X-RAY COMPUTERISED TOMOGRAPHY AND DIRECT CHEMICAL ANALYSIS

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(Received March 25, 1996; accepted May 28, 1996)

The total body fat content of growing rabbits was determined by X-ray computerised tomography (CT) and direct chemical analysis. Serial scans were taken on Pannon White rabbits at the age of 6, 8, 10, 12, 14 and 16 weeks (10 animals per group). A total of seven scans per animal were evaluated, two from the scapular arch, three from the region of the kidney, and two from the pelvic region. The variables of the prediction equations were derived from the X-ray density values of the Houndsfield scale interval between –140 and –50, by adding up the frequencies within intervals of 10. Different models were developed on the basis of the MGLH stepwise procedure and Principal Component analysis. The R^2 values of the estimations varied between 0.76 and 0.90. The costs of the CT procedure were reduced by scanning three animals at a time.

Key words: Computerised tomography (CT), rabbit, prediction, body fat content

In nutritional experiments, the measurement of body composition is usually based on direct chemical analysis and comparative slaughter technique. In rabbit research, carcass composition has been the main subject of investigations (Rao et al., 1978; Ouhayoun, 1983; Petersen et al., 1988; Parigi-Bini et al., 1992; Szendrő et al., 1996). Fewer papers have dealt with total body composition (DeBlas et al., 1977; Fraga et al., 1978; Parigi-Bini et al., 1990; Fekete et al., 1994). The above-mentioned authors used direct chemical determination of body components, which method is unsuitable for detecting changes over the period of experiment. Fekete (1992) published a review about the methods suitable for estimating the body composition of live animals. One of the possible techniques is the use of X-ray computerised tomography (CT).

CT is suitable for the individual examination of tissue layers with the help of X-ray. In the beginning of the 1980s, Norwegian researchers proved that the different tissues of domestic animals can be characterised by non-overriding Houndsfield (HU) value intervals (Skjervold et al., 1981). The whole scale

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spreads from −1000 (no absorption) to +1000 (total absorption), where the 0 point is the density value of water. Romvári et al. (1996) presented a complete description of the method and its applications in rabbit science.

At the Pannon University of Agricultural Sciences the CT Biological Centre started working with a Siemens Somatom DRG type, third-generation instrument in 1990 (Horn, 1991). Since then that instrument has been utilised in several rabbit experiments (Szendrő et al., 1992, 1994, 1996; Fekete et al., 1993, 1994; Romvári et al., 1993, 1994, 1995; Milisits et al., 1996).

The objective of this study was to monitor changes in the body fat content of growing rabbits and to predict the total body fat content based on CT scanning, slaughtering and direct chemical analysis.

Materials and methods

An experiment was carried out at the Faculty of Animal Sciences of the Pannon University of Agricultural Sciences in Kaposvár. Sixty Pannon White rabbits were examined at six different ages (6, 8, 10, 12, 14 and 16 weeks), 10 animals in each age group. The average liveweight of the groups was 1128, 1731, 2174, 2630, 3134 and 3318 g ± 6%, respectively. The CT scans of 8 mm thickness were taken at night after six hours' starvation. The rabbits (three at a time) were fixed in outstretched position, lying flat in a special "container" without anaesthesia.

The animals were scanned from the neck to the end of the femur. As a first step, the length of the vertebral column was determined on a lateral topogram taken from the 5th cervical vertebra to the femorotibial articulation. In each weight category this was divided by 15 to get the distance between the two neighbouring scans. On the basis of our previous research, in the present study eight scans were processed from three body regions. The scapular arch, the region of the kidneys and the pelvic region were cut with 2, 3 and 2 scans, respectively (designations of the scans in the same order are S3, S4, S12, S13, S14, S17 and S18).

The tomogram or CT picture is a 256 × 256-element matrix. Each individual point of that matrix represents a CT value expressed in Houndsfield units (HU) and is called pixel. Because the scan has a thickness, it can be considered a prism characterised by an X-ray absorption value. For the present work only that part of the total Houndsfield scale was used which is the most characteristic of the fat tissue of rabbits. In the X-ray density interval between −140 and −50 HU 10 "HU variables" were formed in each scan [HU7 = (−140)–(−131), HU8 = (−130)–(−121), ... HU16 = (−50)–(−41)] by summarising the neighbouring CT values.

The rabbits were killed humanely after electric stunning, by the method of Blasco et al. (1993), following a 24-hour period of starvation. The weight of the perirenal fat was measured. Finally the total body fat content of rabbits having an empty gastrointestinal tract was determined by direct chemical analysis. The crude fat content was determined by Soxhlet's method, using hydrochloric acid treatment of the samples, after autoclaving the whole body (H.S. No. 6830-66).

The pixel density data were collected from each scan with the CTPC software (Berényi and Kövér, 1991). The HU variables were constructed using a special programme developed by us. The models of the prediction equations are based on MGLH stepwise regression and Principal Component analysis of the SPSS 5.01 package.

Results

The changes found in the crude fat (CF) and perirenal fat (PF) content of rabbits are shown in Fig. 1 as a function of the liveweight of the different groups.

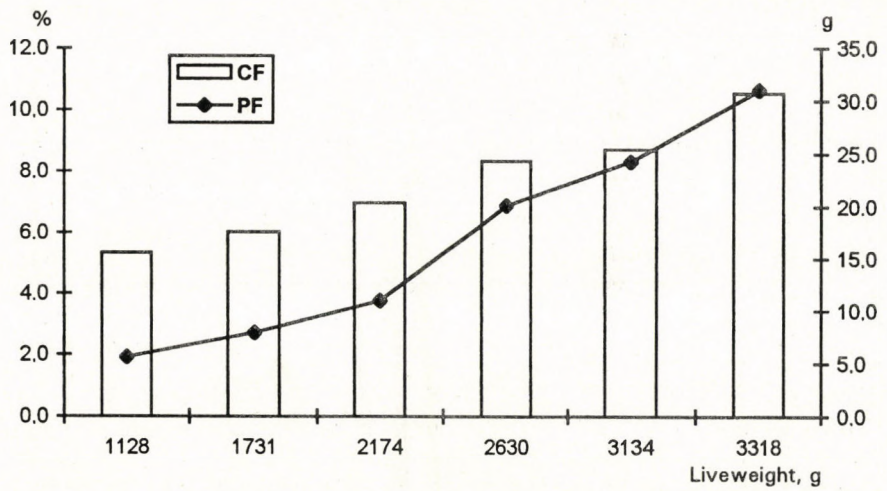


Fig. 1. Crude fat and perirenal fat content of the rabbit groups

While in this liveweight (age) interval the CF content doubled, the weight of PF underwent a 5.5-fold increase in the heaviest group. The correlation between the two values is strong ($R^2 = 0.84$, $P > 0.001$), which means that the weight of the perirenal fat is quite a good indicator of total body fat content.

Subsequently, prediction equations were constructed from the CT data of 60 rabbits in order to predict the total body fat content. The following equation (Eq. 1) is based on the MGLH stepwise regression.

$$y = 4.860242 + 0.013225 \times S4HU11 + 0.008440 \times S18HU12 - 0.004366 \times$$
$$\times S4HU7 + 0.018660 \times S13HU10 - 0.009839 \times S13HU13 - 0.016223 \times$$
$$\times S12HU7 + 0.009809 \times S18HU9$$

where y = fat content (%); S4, S12, S13, S18 = scan position; HU7, HU9, HU10, HU11, HU12, HU13 = HU variables (summarised CT values)

Figure 2 shows the correlation between the observed and the estimated fat content values, where $R^2 = 0.90$ ($P = 0.05$).

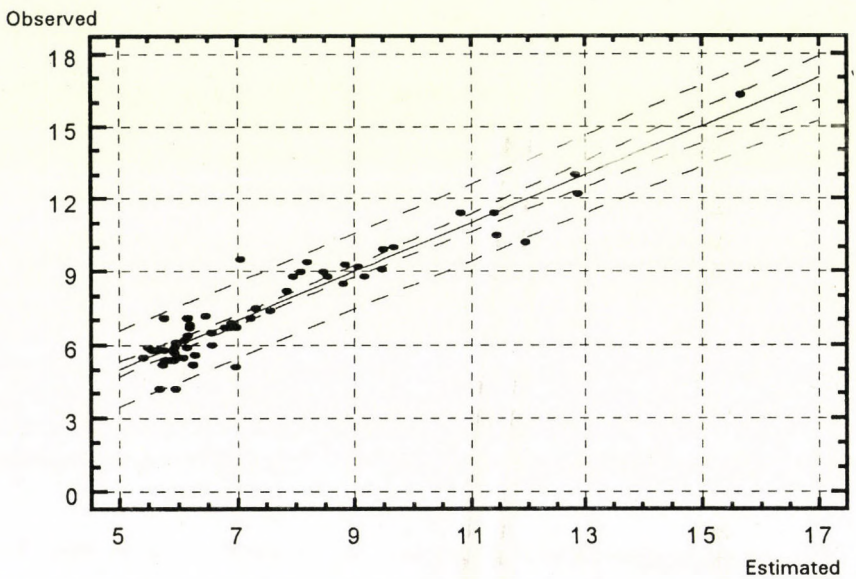


Fig. 2. Correlation between the observed and the estimated fat content (%), as determined by an MGLH procedure

The reliability of the prediction may be lowered by the strong linear connection between the neighbouring HU values [e.g. HU7 = (–140)–(–131), HU8 = (–130)–(–121)]. By applying Principal Component analysis this effect has been diminished in the following equation. The principal components (PCs) of the second equation (Eq. 2) were calculated from the HU variables of scans, taken from the three body regions. Two scans were cut from the scapular arch — 2 × 10 HU variables → 4 PCs (F1-1, F2-1, F3-1, F4-1), three scans were cut from the region of the kidney — 3 × 10 HU variables → 4 PCs (F1-2, F2-2, F3-2, F4-2), two scans from the pelvic region — 2 × 10 HU variables → 4 PCs (F1-3, F2-3, F3-3,

F4-3), altogether 12 PCs were calculated from 70 variables. The eigenvalues of the retained factors exceeded 1.

Equation 2 was built from the above-mentioned 12 PCs by the MGLH procedure.

$$y = 7.567241 - 0.479079 \times F4\text{-}3 - 0.044651 \times F3\text{-}3 - 0.223100 \times F2\text{-}3 + 0.361157 \times F1\text{-}3 - 0.001785 \times F4\text{-}2 - 0.497597 \times F4\text{-}1 + 0.179411 \times F3\text{-}1 - 0.039363 \times F2\text{-}2 + 0.234876 \times F2\text{-}1 + 0.438846 \times F3\text{-}2 + 1.128569 \times F1\text{-}1 + 0.503107 \times F1\text{-}2$$

where y = fat content (%); F1-1, F2-1 ... F4-3 = principal components.

In this case the correlation is slightly lower than in the first model (Eq. 1): $R^2 = 0.81$ ($P = 0.05$).

To reduce the number of variables (from 12 to 3), the equation was recalculated by the stepwise procedure (Eq. 3). Although the R^2 value decreased to 0.76, the correlation was still strong enough.

$$y = 7.567241 + 1.191475 \times F1\text{-}1 - 0.692985 \times F4\text{-}1 + 0.841294 \times F1\text{-}2$$

where y = fat content (%); F1-3, F3-2, F4-1, F4-3 = principal components.

The final model was built from the principal components including the liveweight as independent variables by the stepwise procedure. The form of the fourth equation (Eq. 4), where $R^2 = 0.81$ ($P = 0.05$), is the following:

$$y = 4.498941 + 0.553323 \times F1\text{-}3 - 0.426193 \times F4\text{-}1 + 0.747704 \times F1\text{-}1 + 0.523124 \times F3\text{-}2 - 0.320701 \times F4\text{-}3 + 0.001321 \times LW$$

where y = fat content (%); F1-3, F3-2, F4-1, F4-3 = principal components; LW = liveweight.

Figure 3 shows the correlation between the observed and the estimated fat content based upon the above-mentioned equation.

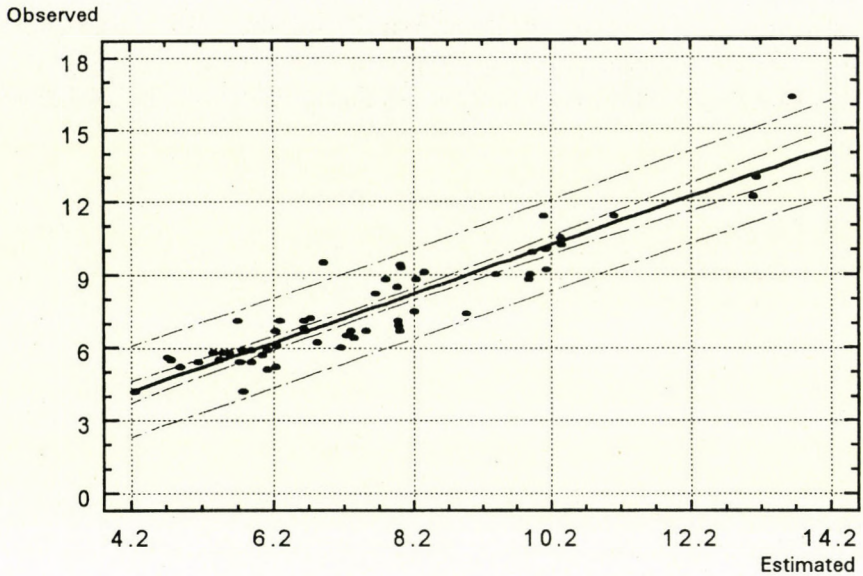


Fig. 3. Correlation between the observed and the estimated fat content (%), as determined by principal component analysis

Discussion

The goal of this study was to develop an *in vivo* method for predicting the total body fat content of rabbits on the basis of CT scanning. The correlations found between the results of chemical analysis and the pixel density data of the pictures are high enough for estimating the fat content. While the MGLH gives a higher R^2 (Eq. 1), the Principal Component analysis (Eq. 4) seems to be more robust. Similar results were reported for chickens by Bentsen and Sehested (1989) and Romvári et al. (1994). For practical purposes, the predictability of the method can be improved by the use of higher numbers of animals. This would enable the detection of changes in body composition in special feeding experiments. The measuring costs can be reduced by scanning three animals at a time. In conclusion, X-ray computerised tomography is a powerful non-invasive method for measuring the body composition.

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THE ROLE OF FEED PROTEIN QUALITY IN REDUCING ENVIRONMENTAL POLLUTION BY LOWERING NITROGEN EXCRETION III. STRATEGIES OF FEEDING: A REVIEW

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To meet the requirements of pigs a better supply of amino acids can be achieved by using ileal digestibility data of feed ingredients in least-cost feed formulation. Digestibility of amino acids can be improved by addition of multi-enzyme products, antimicrobial additives and probiotics. Protein accretion may be positively influenced by beta-adrenergic agonists. An adequate ratio of protein to dietary energy is essential to achieve optimal protein utilization. Nitrogen output can be minimized by improving feed efficiency and by eliminating antinutritional substances with adequate processing. The use of the ideal protein concept helps to minimize nitrogen excretion. Management and animal hygiene also have a significant impact on the overall nitrogen balance in animal production.

Key words: Protein quality, reduction of nitrogen excretion, feeding strategies

The importance of dietary protein quality and of the proper choice of appropriate methods for monitoring protein adequacy have been emphasized in previous articles (Hegedűs, 1993*a*, 1993*b*). However, feeding strategies may also have a strong influence on protein utilization and nitrogen excretion. This article attempts to discuss the possible principles of improving protein absorption, feed efficiency and protein accretion in monogastric animals by means of feed formulation using ileal digestibility data, utilizing the ideal amino acid composition concept as well as by the use of feed additives and phase feeding.

Adequate supply of amino acids based on ileal digestibility data

The excretion of unnecessary nitrogen by the animal is mainly due to digestive inadequacy, consumption of several amino acids in excess of the requirements, and inefficient utilization of amino acids for protein accretion. Digestibility

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of dietary protein or amino acids is the main factor in nitrogen excretion via the faeces, which refers to the absorbable portion of the nitrogen intake. Protein as well as amino acid digestibility can be measured by the method of ileal or faecal analysis (Zebrowska, 1975; Gundel and Babinszky, 1988; Kubovics et al., 1989; Szelényiné, 1995).

The least-cost formulation of rations in many countries is still based upon the content of crude protein and total content of some essential amino acids (lysine, methionine plus cystine, threonine and tryptophan). However, the absorbable amino acid content more precisely shows the adequacy of the feed to meet the animal's amino acid requirements (Van Leeuwen and van Kempen, 1993).

The amino acid digestibility coefficients determined by the method of ileal analysis (mostly those of tryptophan and threonine) are in most cases lower than those determined by the faecal method. The ileal amino acid digestibility values have been shown to be more sensitive for detecting differences in amino acid digestibility of processed feedstuffs (toasting, heat sterilizing); however, the dietary levels of crude protein, crude fibre and crude fat may affect them (Haydon et al., 1984; Imbeah and Sauer, 1991; Sauer et al., 1991; Li et al., 1993). Carbohydrates and especially the cell wall components affect ileal amino acid digestibility negatively. At low dietary protein levels the apparent digestibility is lower than at higher levels (Den Hartog et al., 1989). The additive nature of ileal digestibility values must also be confirmed to enable their reliable use in least-cost ration formulation.

The precise knowledge of digestible amino acid supply, with especial regard to the limiting amino acids, is important for adjusting the composition of the ration so that it can meet amino acid requirements, rectify deficiencies, and avoid surpluses (Rudolph et al., 1983; Knabe et al., 1989).

Improving digestibility by the use of feed enzymes

Although the concept of adding enzymes to animal feeds to improve their nutrient digestibility and utilization has long been known, the results obtained by its use have been inconsistent and sometimes discouraging (Werner, 1982).

The use of non somatic enzymes capable of degrading soluble non-starch polysaccharides (β-glucanase, pentosanase, xylanase, pectinase) was shown to be more beneficial than that of somatic enzymes (proteases, α-amylase and lipase), which are in most cases abundant in the gastrointestinal tract of the animal. An exception is the newly weaned pig, where digestive enzyme insufficiency is well established (Szabó, 1981; Bedford et al., 1992).

Enzyme additions for degradation of non-soluble non-starch polysaccharides (hemicellulase, cellulase) have produced mainly inconsistent results.

In recent years, advances in biotechnology have brought along new methods for producing enzymes using efficient microbes (e.g. *Aspergillus niger*, *Bacillus subtilis*, *Bacillus licheniformis*, *Trichoderma longibrachium*, *Trichoderma viridae*, etc.). These commercially available multi-enzyme products may have benefits in wheat-, barley-, soy-based piglet feeds, in wheat-, maize-, soy-based piglet diets, in high-barley grower and finisher pig diets, and in wheat-, barley-based broiler diets (Graham et al., 1986; Harker, 1991; Classen et al., 1991).

Activity loss during storage and stability during pelleting of feed additive enzymes are often subject to debate; however, stabilized products are also available.

Feed additives to improve amino acid utilization and growth rate

Beta-adrenergic agonist compounds (e.g. clenbuterol, cimaterol, ractopamin, isoprotenerol, salbutamol), given orally or parenterally, have a positive effect on skeletal muscle accretion and a negative effect on fat retention in adipose tissue depots (nutrient repartitioning agents). Their structures are related to epinephrine, a hormone synthesized and secreted by the adrenal medulla under the control of the sympathetic nervous system (Baker et al., 1984). Beta-adrenergic agonists have been shown in numerous experiments not only to promote growth, but also to increase protein utilization and to reduce urinary N-excretion (e.g. DeB et al., 1989; Etherton and Louveau, 1992); however, the sensitivity of the animal to heat and transport stress may be higher.

Reduction of gastrointestinal microflora by nutritive non-absorbable antibiotics or other antimicrobial chemotherapeutics results in growth promotion (Wisek, 1978). Other effects of antimicrobial additives are: reduction of ammonia as well as microbial toxin production in the intestine, thinning of the intestinal wall resulting in enhanced efficiency of nutrient absorption (Fethiere and Miles, 1987). The most pronounced effect can be observed in weanling pigs. It is assumed that antimicrobials may slightly improve digestibility, but the efficiency of protein accretion is unaffected (Roth and Kirchgessner, 1993). The beneficial effect of antimicrobial agents on nitrogen excretion is a consequence of improved feed efficiency (lower feed intake, higher weight gain), rather than of improved protein utilization.

Antimicrobial feed additives have proved to be the most consistently effective growth promoters even in recent years. However, there is a trend to expel nutritive antibiotics from feeds, which can be attributed to the pressure exerted by health-conscious activist groups and the marketing policy emphasizing the image

of healthy foods, rather than to the real danger presented by the problem of growing bacterial resistance to antibiotics (Smith, 1993; Lamming, 1993).

Probiotics are used to improve the gastrointestinal microbial profile. Most probiotic products contain living cells of *Lactobacillus*, *Streptococcus*, *Bacillus*, and *Saccharomyces* strains, which are able to regulate the composition of gastrointestinal microflora and to prevent the invasive action of pathogenic strains (Vanbelle et al., 1990; Gedek, 1994). The best response to probiotics can be achieved just after weaning, and the effect declines with age. Generally a lower incidence of morbidity and mortality can be observed. However, the response of starter pigs to probiotics seems to be variable, depending on the product and the circumstances of application. Daily weight gain and feed efficiency are sometimes unaffected by the use of probiotics. The potential of probiotics in reducing N-excretion is limited, as they have only an indirect effect on protein utilization.

Optimal ratio of protein and amino acids to energy for maximum nitrogen accretion

Pigs and poultry are considered to eat in order to meet their energy requirements and, therefore, energy content of the diet is the main factor controlling their voluntary feed intake. It has been suggested that nutritional requirements be expressed in relationship to the energy content of the diet (ARC, 1981). However, there is little agreement in the literature regarding the optimum ratio of protein and lysine to energy. Lysine is most often the first limiting amino acid in pig rations. The range in suggested values for weanling pigs is 0.67–0.99 g lysine/MJ digestible energy. The values may be affected by age, body weight, genotype, sex, differences in feed ingredients, dietary protein, and energy level (Nam and Aherne, 1994).

The balance between protein and energy supply is essential for minimizing the use of protein for energy catabolism in the body. If the carbon chain of protein is catabolized for energy, the amino group of amino acids will be excreted in the urine as urea. The long-recognized protein-sparing effect of carbohydrates and fats can be explained by their energy supply for protein deposition and by their role in saving protein being catabolized as energy source.

The optimal ratio of protein to energy has been comprehensively studied in feeding experiments in the past to determine energy and protein requirements (e.g. Gundel and Szentmihályi, 1983). As the optimal ratio of protein to energy in the feed decreases during fattening, less protein and more energy will be needed in the feed during the finishing period.

Improvement of feed efficiency

Improvement in overall reduction in the feed to gain ratio would reduce nitrogen excretion. For each 0.1 percentage unit decrease in feed to gain ratio there was a 3% decrease in nitrogen output in growing-finishing pigs (Kornegay, 1994).

Various strategies can be applied to achieve a better feed efficiency: improving the animals' genetic potential for a higher capacity of protein accretion, proper formulation of rations using highly digestible ingredients, and applying appropriate processing and feeding methods. All feed additives capable of enhancing digestibility and protein utilization may be utilized for improving feed efficiency and thus decreasing nitrogen output.

The nitrogen output of grower-finisher swine of 20–100 kg body weight is in the practice about 5.5 kg instead of the theoretical 2.5 kg (DLG, 1993). If the feed to gain ratio improves from 3.3 to 3.0 kg/kg, the nitrogen output can be reduced by 0.7 kg N (12.5%). If the dietary protein level is decreased from 17.5 to 15.5 g/100 g, an additional 0.7 kg (12.5%) reduction in nitrogen output can be achieved. With supplementation of limiting amino acids and phase feeding another 1,6 kg N (30%) can be spared. The percentage of the total nitrogen output which can be theoretically spared is thus 55%, compared to the present field situations.

The nutrient content of poultry feeds is regarded to be better adjusted to the requirements compared to swine feeding, therefore it is more difficult to achieve a lower nitrogen output. Reduction of the dietary protein level of poultry feeds may result in lower performance and higher abdominal fat irrespective of amino acid supplementation.

Elimination of antinutritional factors by adequate processing

Protease inhibitors, lectins, tannins, alpha-amylase inhibitors are present in most legume seeds, and exert a negative influence on the ileal or faecal digestibility of amino acids (Sauer and Ozimek, 1986), leading to increased nitrogen output. Steam treatment (toasting), extrusion, hydrothermal treatment, infrared irradiation, dry fractionation and the use of chemicals may effectively eliminate antinutritive substances from the feed. The quality control of feed ingredients potentially containing antinutritive factors is essential for maintaining high production with minimum loss of nitrogen.

Improvement of protein utilization — Ideal protein concept

The importance of protein quality and particularly the balance between essential and nonessential amino acids, as well as the practical utilization of the ideal protein concept were recognized long ago and have been discussed by many authors (e.g. Harper and Rogers, 1965; Eggum, 1973; Kralovánszky et al., 1988; Fuller et al., 1989; Marbery, 1992; Goihl, 1992). The quality of a protein can be assessed by comparing it with the ideal protein, which has an optimum amino acid pattern and assumed to be fully utilizable for the animal. The optimum amino acid pattern is expressed as ratios to lysine.

The ideal amino acid composition concept can be applied to the ileal digestible amino acids as well. Expressing both amino acid requirements and dietary amino acid supply in "ideal ileal digestible protein" may help to improve protein utilization and reduce nitrogen excretion (Lenis et al., 1993).

Protein utilization can be improved by adjusting the amino acid composition of the feed to a composition similar to that of ideal protein. To eliminate deficiencies is primarily important for optimum performance, and to cut surpluses is important for minimizing nitrogen excretion in the urine. If the amino acid composition of the ideal protein can be approximated, the protein level of the diet can be slightly reduced, in most cases by 2 percentage units (Jongbloed and Lenis, 1992), without a reduction in the animal's performance. Reduced protein intake and improved utilization of amino acids result in lower urinary nitrogen output.

Numerous experiments have been conducted to reveal the optimum amino acid supplementation of feeds depending on their ingredients, protein and energy levels (e.g. Rosell and Zimmermann, 1984; Wahlstrom et al., 1986; Iben and Leibetseder, 1987; Haydon et al., 1989; Goihl, 1990; Kornegay et al., 1993). Although a considerable amount of work has been reported on amino acid nutrition, it is difficult to obtain precise amino acid requirement values and thus optimum levels of supplementation because of the numerous factors affecting their utilization (Hegedűs, 1992).

Lowering protein level of the feed with amino acid supplementation

Numerous experiments have been carried out to find the proper balance between protein level and amino acid composition of the diet while maintaining optimum weight gain (e.g. Eggum, 1980; Russel et al., 1987; Gatel and Grosjean, 1992; Hansen et al., 1993; Markert et al., 1993; Page et al., 1993; Roth et al., 1993; Kirchgessner et al., 1994). Growth and feed efficiency of swine have been shown to be satisfactory when slightly lowered protein levels are used with con-

comitant supplementation of limiting amino acids (mostly lysine, methionine, tryptophan, threonine); however, optimum performance is not achieved when the animals are fed very low protein diets irrespective of amino acid supplementation. Poultry are more sensitive in this respect than swine.

It seems that a minimum amount of intact protein or peptides is necessary for optimum animal performance (Keshavarz, 1991; Jensen, 1992). This may be due to the more rapid transfer of small peptides into the enterocyte during absorption, as compared to that of free amino acids. Peptide transfer was shown to be less energy dependent during absorption.

The efficiency of protein utilization can be improved and the urinary excretion of nitrogen reduced by lowering the dietary protein level; however, the daily weight gain of the animal may decline. According to the results of Jost (1993), diets for piglets containing only 16% crude protein or less result in an unprofitable growth depression in spite of supplementation with lysine, methionine plus cystine, threonine and tryptophan. On the basis of minimum nitrogen emission and acceptable weight gain of piglets, the optimum crude protein content in the diet with 13.9 MJ DE energy content, without antimicrobial growth promoter, was 17%, if the first limiting amino acid (lysine) was supplemented.

Phase feeding

Phase feeding is a way to meet the amino acid needs of growing and finishing pigs more precisely. As the protein requirement relative to energy decreases during fattening, ideally a weekly adjustment of dietary nutrient content would be necessary. Thereby the total amount of nutrients could be reduced (Kornegay, 1994). The multi-phase feeding proposed by Boisen (1993) utilizes two complete feeds, which corresponds exactly to the requirement for ideal protein relative to energy at 25 and 100 kg liveweight, respectively. The starting feed should then be gradually replaced by the finishing feed throughout the growth period. The starting feed is high in energy as the energy intake is the limiting factor for protein deposition until about 50 kg liveweight. To obtain minimal N-excretion and optimum weight gain, the ileal digestibility of amino acids and the amino acid composition of the ideal protein must be known. However, it is difficult to perform weekly adjustments in feed composition, and such changes may affect the eubiosis of the gastrointestinal microflora.

Intensity of fattening

If the weight gain is high, the portion of maintenance protein requirement will be lower within the total nitrogen requirement. Consequently, the higher the intensity of fattening, the lower the total amount of nitrogen excretion per product (meat, eggs, etc.). At a lower intensity of feeding the daily nitrogen excretion per animal is less, but the duration of fattening will be longer, resulting in a higher nitrogen output related to the end-product (Kirchgessner and Roth, 1993).

Animal hygiene

Unfavourable environmental conditions (housing, temperature, air quality, infections) are regarded as stress factors for the animal, which adversely affect weight gain and feed efficiency. Diseases can considerably increase the animal's protein turnover and, as a consequence, the extent of nitrogen excretion via the urine. The maintenance protein requirement of the sick animal may increase to 2–3 times the resting level (Kienzle, 1992). Parasites may lead to direct protein loss. Absorption of nutrients may decrease when scours occur, with a concomitant increase of the faecal nitrogen loss.

Pig feeds often contain trichothecene mycotoxins which have been shown to impair the animals' immune system (Rafai et al., 1995*a*). Weight gain, feed intake, the extent of feed refusal, and the protein metabolism of pigs were negatively affected by different oral doses of T-2 toxin in controlled feeding experiments (Rafai et al., 1995*b*).

The prevention of infectious and production diseases by adequate management has a significant impact on the overall nitrogen balance of fattening.

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A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC (HPLC) METHOD FOR THE SIMULTANEOUS DETERMINATION OF NITRITE AND FORMALDEHYDE FROM FOODS

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A high-performance liquid chromatographic (HPLC) procedure with fluorometric detector has been developed for the determination of nitrite and formaldehyde from foods by the use of hydralazine. Hydralazine reacts with nitrite and formaldehyde under acidic conditions in boiling water-bath for 15 min to form tetrazolo-(5,1-a)-phthalazine (Tetra-P) and triazolo-(3,4-a)-phthalazine (Tri-P) quantitatively. Without extraction, the determination of Tetra-P and Tri-P was simple, specific, sensitive and reliable over the range of 0.003–0.3 ppm of sodium nitrite and 0.02–0.4 ppm of formaldehyde. This procedure using hydralazine is one of the most useful methods for routine analysis of nitrite and formaldehyde in foods, biological fluids and ambient waters.

Key words: Formaldehyde, nitrite ion, hydralazine, determination, high-performance liquid chromatography (HPLC), foods

Hydralazine (1-hydrazinophthalazine, HP) is an effective depressor of hypertension. It reacts with nitrite and formaldehyde in acidified medium to give tetrazolo-(5,1-a)-phthalazine (Tetra-P) and triazolo-(3,4-a)-phthalazine (Tri-P) (Fig. 1). These transformations could be applied to the development of a simple and sensitive method for the determination of nitrite and formaldehyde in foods by means of high-performance liquid chromatography.

For nitrite analysis, colorimetric methods using sulphanilic acid (Usher and Telling, 1975), or sulphanilamide (Standard Methods, 1980) have generally been used. Gas-chromatographic (GC) methods using o–phenylenediamine (Akiba et al., 1973), or aromatic primary amines for the Sandmayer reaction and HPLC of nitrite itself without any derivatization (Thayer and Huffaker, 1980) have been reported. Colorimetric methods lack specificity, which is a characteristic crucially important for analysis because the sample is often turbid and slightly coloured. GC methods generally require complex pretreatments, and the methods using HPLC for nitrite itself have unsatisfactory detection limits. On the other hand, for

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the analysis of formaldehyde, colorimetric determination has generally been used by measuring the condensation products with acetylacetone-ammonium acetate (Standard Methods, 1980), 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Avigad, 1983), or chromotropic acid (Chafetz et al., 1984). Colorimetry is not necessarily reliable because the samples are sometimes opaque and coloured. A GC method using 2,4-dinitrophenyl-hydrazine (Spreitzer and Jager, 1990) or cyanide ion (Improta et al., 1984), and HPLC of the condensates with acetylacetone-ammonium acetate (Okamoto and Yamada, 1981) or with 2,4-dinitro-phenyl-hydrazine (Olson and Swarin, 1985) have also been reported. However, these require rather complex pretreatments such as the extraction of a derivative, and drying and concentration of the solution prior to analysis.

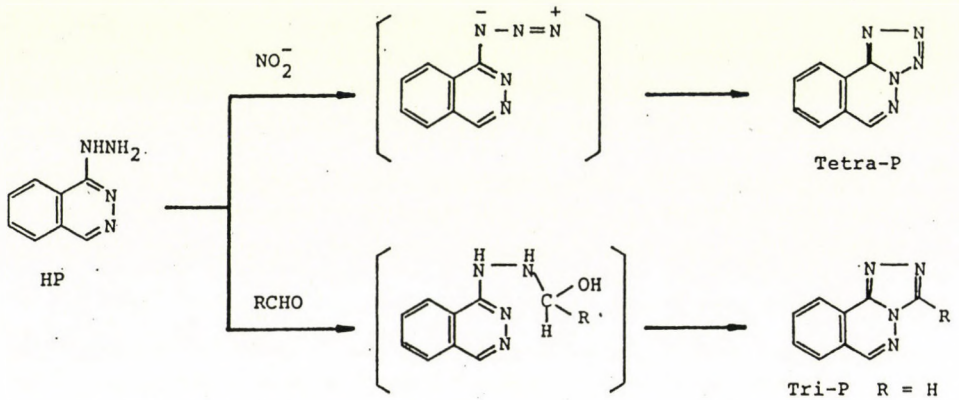


Fig. 1. Reaction scheme of nitrite and/or formaldehyde with hydralazine

The use of a HPLC method for the determination of sodium nitrite and formaldehyde is described in this paper. The method was originally published by Noda et al. (1980, 1986). The procedure used for the preparation of food samples has been developed by us.

Materials and methods

Materials

Chemicals. Reagent-grade HP-HCl was purchased from Sigma (St. Louis, USA), reagent-grade sodium nitrite (analytical grade) from Reanal (Budapest, Hungary), and reagent-grade formaldehyde 35% (analytical grade) from Reanal (Budapest, Hungary). Acetonitrile for the mobile phase was of HPLC grade from Carlo Erba (Milan, Italy). Other chemicals used were of analytical grade.

Apparatus and HPLC conditions. The prepared food samples were injected through a 20 µl loop (Rheodyne, USA) for analysis on a reversed-phase silica column (Nucleosil-7-C18 2 × 250 × 3.9 mm I.D.) (Macherey-Nagel, Düren, Germany) at ambient temperature. The mobile phase (pH cca 4.5) was 30% acetonitrile in 0.05 M KH_2PO_4 pumped by a Waters Model 501 HPLC pump at a flow rate of 1.0 ml/min. Detection was carried out using a Waters 470 type spectrofluorometric detector at 237 nm (excitation) and above 370 nm (emission) wavelengths. The strengthening of the detector was 100 times.

Procedures

Standard solution. To prepare the standard solutions, sodium nitrite and formaldehyde were dissolved in distilled water. For the recovery study, meat samples were spiked with various amounts of sodium nitrite and formaldehyde in the ranges of 3.0–300.0 ppb and 20.0–400.0 ppb, respectively.

Sample preparation. A 100 ml solution of the food extracts was prepared from 5.0 g of food samples according to the procedure described earlier (Csiba et al., 1995*a*, 1995*b*). Accordingly, the matrix materials were removed by the use of 1.0 ml volumes each of Carrez I and Carrez II solutions, respectively.

Determination of sodium nitrite and formaldehyde. A 4.0 ml aliquot of HP hydrochloride (HP-HCl) solution (40 mg in 200 ml of 0.2 M HCl) was added to 4.0 ml of food extracts. The mixture was heated in a boiling water-bath for 15 min. To the cooled reaction mixture, a 4.0 ml volume of acetonitrile was added. The mixture was passed through a filter-paper, if necessary, and 20 µl aliquots were injected directly for HPLC.

Calculations. The concentrations of the sample solutions were determined by interpolation from calibration curves, which were constructed by plotting the peak areas of Tetra-P and Tri-P versus the concentrations of sodium nitrite and formaldehyde (0.003–0.3 ppm and 0.02–0.4 ppm, respectively).

Results and discussion

The test system applied was sodium nitrite and formaldehyde in 2.5 µg/litre concentrations in distilled water. The blind system consisted of distilled water alone. Hydralazine hydrochloride was added to these systems according to the method described above.

The blind and the spiked chromatograms of medicinal plants are presented in Figs 2 and 3. In Fig. 3, the first and second large peaks are Tri-P [Retention time (Rt) = 12.9 min] and Tetra-P (Rt = 15.6 min) from formaldehyde and sodium nitrite, respectively. The two small peaks before the Tri-P are of unknown origin.

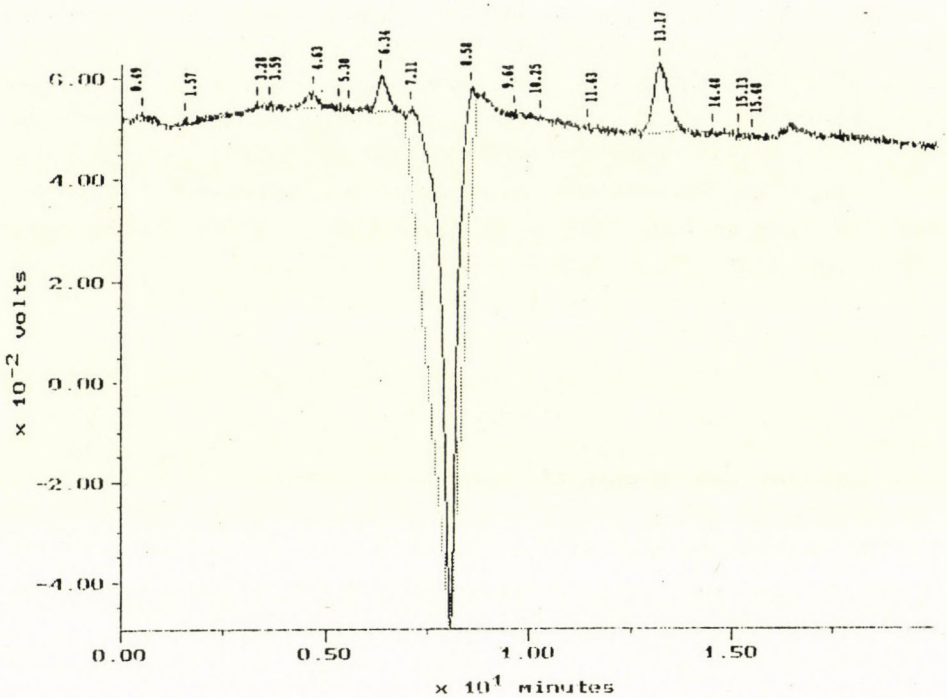


Fig. 2. HPLC chromatogram of the reaction mixture of HP-HCl in water. HPLC conditions: loop volume, 20 µl; column, Nucleosil C18, 2 × 250 × 3.9 mm I.D; mobile phase, 30% acetonitrile in 0.05 M KH_2PO_4; temperature, ambient; flow rate, 1.0 ml/min. Detection, fluorescence, excitation = 237 nm, emission = 370 nm, amplification: 100 times

Analytical data

Linearity. In the concentration range studied (0.15–15.0 ng/ml for sodium nitrite and 1.0–20.0 ng/ml for formaldehyde), the detector response was found to be linear for both peak area and peak height measurements.

The calibration equations for sodium nitrite determinations are:

$Y = 1.72\ X$, where Y = detector response in V.s,
X = sodium nitrite concentration in µg/litre;

and/or

$Y = 0.086\ X$, where Y = detector response in V.s,
X = sodium nitrite concentration in µg/kg of sample.

The calibration equations for formaldehyde determinations are:

$Y = 1.56\ X$, and/or $Y = 0.078\ X$, where the meaning of symbols Y and X is as given above.

Limit of detection. The least possible detectable concentrations were found to be 0.1 ng/g of sodium nitrite and 0.5 ng/g of formaldehyde, calculated as three times the standard deviation of the noise.

Limit of quantitation. By the sample preparation method the limits of quantitation were 3.0 ng/g for sodium nitrite and 5.0 ng/g for formaldehyde.

Recovery. The recovery was found to be 95 ± 2% in the 20.0–200.0 ng/g concentration range for the whole process for sodium nitrite and formaldehyde, respectively.

Precision. The intra-day reproducibility was studied. The results showed good agreement, with a variation coefficient of 0.2% at 10.0 μg/litre sodium nitrite and/or formaldehyde concentration.

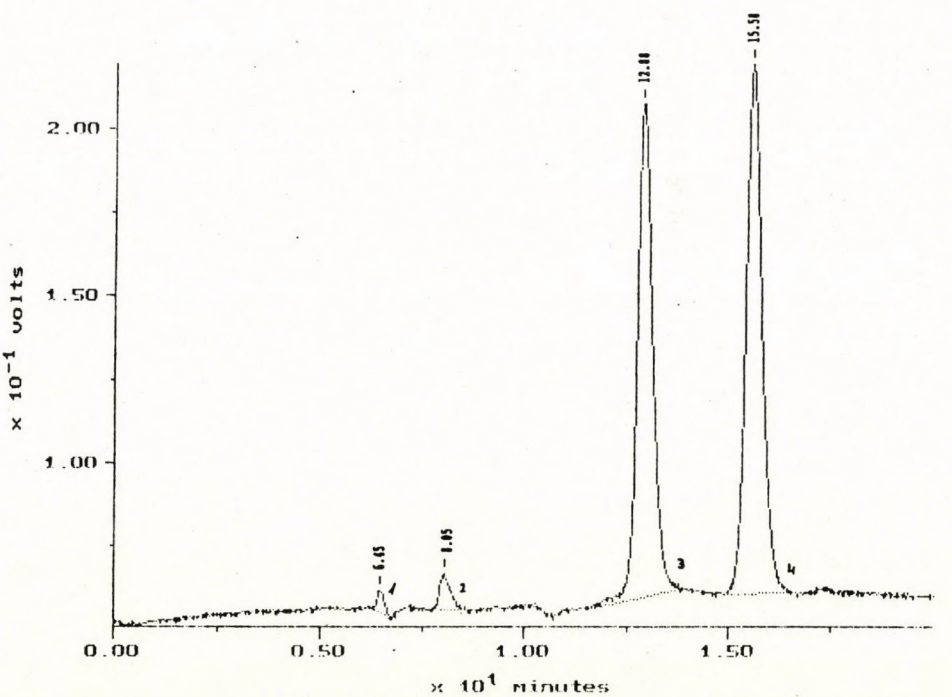


Fig. 3. HPLC chromatogram showing medicinal plants spiked with 50 ng/g of sodium nitrite and formaldehyde. Peaks: 1,2 = unknown; 3 = Tri-P; 4 = Tetra-P. HPLC conditions as in Fig. 2

Practical application

Figure 4 shows the HPLC chromatogram of a sample made from "Milanese" cold meat cuts. The sodium nitrite peak is three times higher than the formaldehyde one.

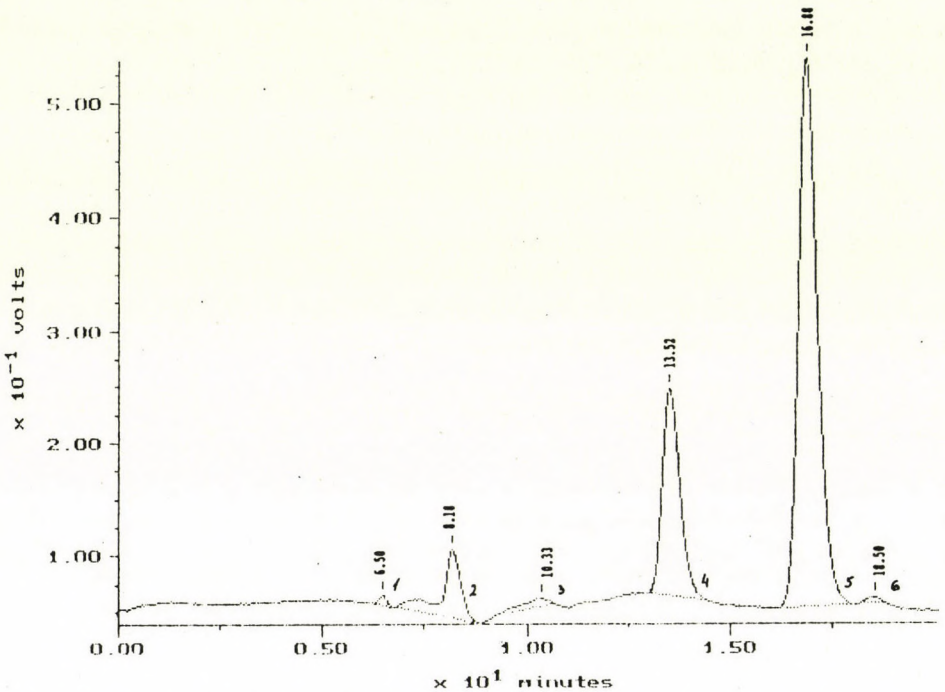


Fig. 4. HPLC chromatogram of a "Milanese" cold meat sample. Peaks: 1,2,3,6 = unknown; 4 = Tri-P; 5 = Tetra-P. HPLC conditions as in Fig. 2

The results of determination of sodium nitrite and formaldehyde in medicinal plants, milk, cold cuts, sausages, and canned meat products are presented in Table 1.

The roots of medicinal plants contain nitrite in small concentrations. This nitrite may derive from the soil. The nitrite content of cold meat cuts and sausages comes from the pickle medium.

Very small concentrations of formaldehyde can occur also in biological systems as endogenous pool. Exogenous formaldehyde occurs in the environment (drinking water, clothes, drugs, containers, etc.) and may get into food samples.

Table 1

Analytical results obtained by the proposed method

Sample	Sodium nitrite mg/kg	Formaldehyde mg/kg
Medicinal plants		
Yarrow *(Achillea millefolium)*	0	4.6
Common comfrey *(Symphytum officinale)*	0	11.9
Althea root *(Althaea)*	4.2	7.2
Hawthorn *(Crataegus sp.)*	0	3.6
Mistletoe *(Viscum album)*	1.2	6.8
Raw milk	1.0	0.5
Cold meat cuts		
Cold meat cuts "Milanese"	29.5	10.0
Bologna sausage	57.1	10.0
Miscellaneous cold meat cuts	78.7	11.0
Sausages		
"Casino" sausage with red pepper	11.6	3.1
"Casino" sausage	11.6	2.5
"Cserkész" sausage	11.1	2.9
Canned meat		
Liver paste (pâté)	3.4	1.7
Canned fish	0.4	3.2
Luncheon meat	2.6	0.8
Canned fish in tomato	0.5	6.2

An alternative analytical procedure based on the same reaction but done by GC was described by Tanaka et al. (1981). Their method gave very satisfactory results, i.e. very high recoveries of nitrite from foods, milk and blood, and excellent detection limits [0.02 ppm, Gas Chromatograph with Flame Ionization Detector (GC-FID); 0.3–4.0 ppb, Gas Chromatograph with Electron Capture Detector (GC-ECD)]. The sensitivity of the GC-ECD method is comparable to that of Noda's method adapted by us. However, the GC method requires more complex sample preparations (extraction, drying, evaporation, etc.), none of which is necessary when using the HPLC procedure adapted by us. The most remarkable advantages of the HPLC method are concluded to be its high sensitivity and simple

sample preparation procedure. In conclusion, the described method is considered to be one of the most useful procedures for the routine analysis of nitrite and formaldehyde in food and environmental samples.

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MAST CELLS IN LYMPH NODES OF COWS INFECTED BY BOVINE LEUKAEMIA VIRUS

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The number of mast cells (MCs) was determined in the prescapular lymph nodes of 109 animals seropositive to bovine leukaemia virus (BLV). The number of MCs was studied in relation to a variety of pathological reactions affecting lymph nodes. A significant increase of MCs was found in T zonal hyperplasia, B zonal hyperplasia, and mixed hyperplasia ($P < 0.01$) as compared to the control group. There was no significant increase in pulp proliferation and atrophy of the lymph nodes. MCs were mainly found in the medullary cords and sinuses. The possible role of MCs in the regulation of immune responses to enzootic bovine leukaemia (EBL) is discussed.

Key words: Cattle, bovine leukaemia virus (BLV), lymph node, mast cells (MCs)

Mast cells (MCs) are widely distributed throughout the tissues of the body, including the lymph nodes (Parwaresch et al., 1985). MCs are crucial in the development of immediate hypersensitivity reactions. They are also implicated in a wide range of biological responses in which IgE lacks any apparent role. These include a variety of diseases associated with chronic inflammation, as well as tissue remodelling and pathological fibrosis (Swieter et al., 1993). During these pathological processes, mast cells increase in number at sites of tissue injury. They are characterised by metachromatic granules and by the synthesis of a number of well-recognised pharmacologically potent mediators (Gordon et al., 1990).

The distribution of reactive MCs in the lymph nodes of bovine leukaemia virus (BLV) seropositive cows has not been studied yet. The results of serological testing for this virus was reported for two cows with mast cell tumour. In one case, a cow with mast cell tumour had antibodies to BLV (Stephens and Mullowney, 1986; Shaw et al., 1991). The relationship of BLV to the development of mast cell tumours is not well understood.

Our previous study divided pathomorphological reactions of the lymph nodes of BLV-infected animals into individual groups (Levkut et al., 1994) based

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upon of the criteria used for the examination of human lymph node biopsies (Levkut et al., 1993). The purpose of this study was to examine and quantify MCs in the lymph nodes of BLV-infected animals.

Materials and methods

Animals. Prescapular lymph nodes from 109 cows seropositive to BLV, and from 10 negative control cows were used in this study. The majority of the animals belonged to the Black Spotted and Slovak Spotted breeds (4–6 years of age).

Detection of BLV antibodies. An immunodiffusion test (Bioveta, Nitra, Slovakia) was used for detecting BLV antibodies.

Processing of histological material. The material removed was processed using standard procedures, i.e. fixed in 10% neutral formalin and embedded in paraffin. Sections of a thickness of 5–6 μm were stained by Giemsa and naphthol AS-D chloracetate esterase reactions.

Evaluation criteria. Mast cells were evaluated in the medullary cords and sinuses of lymph nodes. They were counted in 100 light-microscopic visual fields at a magnification of × 1000.

Statistical analysis. Statistical significance of the data was evaluated by Student's *t*-test.

Results

Macroscopic examinations of 109 slaughtered animals did not reveal any neoplastic processes or parasitic invasion of the predilective organs. Some prescapular lymph nodes showed a moderate enlargement. Visible follicular hyperplasia at the cut surface or an indistinct structure was observed in the enlarged nodes. Histological examination, which divided the pathomorphological reactions of the lymph nodes into five groups, was reported earlier (Levkut et al., 1994).

The histological evaluation of MCs in the medullary cords and sinuses of the prescapular lymph nodes of 109 animals is shown in Table 1. A significantly increased number of MCs was observed in T zonal hyperplasia, B zonal hyperplasia, and mixed hyperplasia (all groups $P < 0.01$). No statistical changes were found in pulp proliferation and atrophy of the lymph nodes.

MCs were loosely scattered in the medullary cords and sinuses. Infiltration of the T zone was rare, and MC infiltration of the lymph follicles was absent. MCs often contained many metachromatic granules, enabling easy identification of these cells by Giemsa staining. Naphthol AS-D chloracetate esterase reaction

showed the same number of MCs. Cells with many granules were often round or oval in shape, while those with sparse granules were often elongated. Mitoses were not seen. In some cases eosinophilic infiltration of the sinusoids, medullary cords and T zone was observed.

Table 1

Mast cells in pathological conditions of the prescapular lymph nodes in 109 cows

Pathological conditions	No. of cases	No. of mast cells	
		Mean	± SD
T zonal hyperplasia	52	*25.47	34.57
B zonal hyperplasia	23	*12.09	17.78
Mixed type	18	*22.78	22.32
Pulp proliferation	6	3.33	4.89
Atrophy	10	21.25	24.46
Control group	10	2.36	2.53

* Significantly different from the control group ($P < 0.01$). Values are expressed as mean ± standard deviation (SD) per 100 light-microscopic fields at a magnification of × 1000

Discussion

This study has shown a significant increase in the number of MCs in T zonal, B zonal, and mixed hyperplasia. MCs were mainly scattered in medullary cords and sinuses. Generally, it is significant that the medullary cords and sinuses are those compartments of lymph nodes which normally exhibit an increase of MC cells in chronic nonspecific lymphadenitis, while in the lymph follicles and paracortex, MCs are virtually absent (Horny et al., 1992). In the lymph node reactions mentioned above there were relatively higher numbers of T lymphocytes as compared with pulp proliferation and atrophy of the lymph nodes. T lymphocytes have been shown to play a major role in the increase of MCs via the production of IL-3. Other cytokines produced by T cells such as IL-4, IL-10 and stem cell factor could also be important (Marshall, 1993).

The peripheral lymphocytes of BLV-infected cattle show a higher expression of IgM and IgG molecules as a rule (Fossum et al., 1988; Levkut et al., 1995*b*). The increased number of IgM and IgG molecules could activate the complement system, which has been shown to be a potent liberator of MCs (Par-

waresch et al., 1985); however, the low number of MCs in some cases of T zonal, B zonal, and mixed hyperplasia remains obscure.

In some diseases, depletion of MCs in the tissues is followed by massive antigen–antibody reactions, especially in cases characterised by a high concentration of circulating antibodies. Later, upon restimulation the sensitized T lymphocytes may secrete a specific lymphokine likely to induce MC formation (Parwaresch et al., 1985). Some results suggest that cell-mediated immune responses are responsible for the downward regulation of BLV replication in carrier animals (Sugimoto et al., 1993). The concentrations of serum antibodies to BLV differ by animal, varying from low to high titres (Bause and Schmidt, 1980).

MCs are a source of multifunctional cytokines, which are on induction with the ligand of the c-kit tyrosine kinase receptor (CD117) (Valent, 1994). The first cytokine bioactivity to be clearly associated with normal MCs was the tumour necrosis factor (TNF-α) or cachectin (Steffen et al., 1989; Gordon et al., 1990). TNF-α is an indicator of cytolytic activity against certain cellular targets, and results in increased antiviral activity (Ramsay and Ramshaw, 1993). The highest concentration of MC-derived cytokines is found in the interstitial spaces, rather than in the circulation. In most instances, the local consequences of MC cytokine release can be expected to be more significant than the systemic effects (Gordon et al., 1990).

Local eosinophilic infiltration, found in some of our cases (Levkut et al., 1995*a*), appears to be induced by cytokines and mediators released by MCs (Horny et al., 1992), but the reason why they were not observed in all cases remains to be clarified. Reactive MC hyperplasia and MC tumours are usually interspersed with eosinophils (Madewell et al., 1984; Horny et al., 1992). The number of MCs was, in individual cases, the same with Giemsa staining and naphthol AS-D chloracetate esterase reaction. This correlation does not show the occurrence of atypical MCs with very few or any metachromatic granules (Horny et al., 1992). From our results it seems that in BLV-infected animals alterations occur not only in lymphocyte populations but also in MCs. Immunological or pathological processes can result in changes in the phenotype of mast cells (Gordon et al., 1990). Long-term alterations could induce MC tumour with eosinophilic infiltration (Shaw et al., 1991). The data suggest that further investigations are needed to accurately define the role of mast cells and to study phenotypic changes in chronic leukaemic processes caused by BLV.

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TISSUE MACROPHAGES OF CALVES AFTER HIGH DOSES OF GLUCAN

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Relatively high doses of the soluble form of fungal glucan (50 mg/kg of body weight) were administered to two clinically healthy calves. Hepatosplenomegaly was not observed. Quantitative and significant qualitative changes were seen in macrophages in the medulla of lymph nodes as compared to the control animals. Both the fibrillar and the granular components in the vacuoles of phagocytosing cells appeared to be positive in the periodic acid–Schiff reaction (PAS); an amorphous component was PAS negative. Quantitative changes occurred also in the expression of IgM molecules on the surface of B lymphocytes. The results indicate that glucan can be used in ruminants also in a single high dose.

Key words: Glucan, calf, lymph node, macrophage, scanning electron microscopy, immunoglobulins

Glucans belong to the modifiers of biological response. These substances of polysaccharide origin occur as components of different bacteria and in the cell walls of yeasts and plants (Chihara, 1984). Glucan has been shown to increase nonspecific resistance to a variety of experimental infections (Buddle et al., 1988), to inhibit tumour growth (Williams et al., 1985), and to exert significant radioprotective effects (Patchen et al., 1989).

The basis of these activities is suggested to be a stimulation of mononuclear phagocytic cells and the resulting increase in the production of interleukin-1 by these cells (Browder et al., 1987; Takeda et al., 1990). This concept is supported by the demonstration of specific receptors for glucan in the membrane of macrophages (Czop and Austen, 1985).

Studies on the effects of polysaccharides on cell-mediated and humoral immunity in animals have yielded discrepant results. Significant changes in the levels of T and B lymphocytes in the blood of animals were observed only after the simultaneous administration of glucans with additional substances (Benda and Mádr, 1991; Benková et al., 1992). Quantitative changes in the expression of im-

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munoglobulins (Levkut et al., 1995) on the surface of blood lymphocytes after the application of glucan have not been studied.

Experiments with glucans have been carried out on laboratory animals at a dose of 10–20 mg/kg of body weight (Lahnborg et al., 1982; Franěk et al., 1992) and on domestic ruminants at a dose of 10 mg/kg of body weight (Benda and Mádr, 1991; Levkutová et al., 1993).

In this work, relatively high doses of glucan (50 mg/kg of body weight) were administered to calves in order to undertake a morphological study of the activity of macrophages in the medulla of lymph nodes, to determine the levels of T and B lymphocytes in the blood, and to study changes in the expression of IgM on the surface of B lymphocytes by scanning electron microscopy.

Materials and methods

Animals. Four 1-month-old calves of the Slovak Spotted breed were used.

Glucan. Soluble beta (1–3) glucan from *Pleurotus ostreatus* (kindly provided by Dr. Szechenyi, Mevak, Inc., Nitra, Slovakia) was diluted in aqua pro injectione. Glucan (4%) was administered to two calves i.m. at a dose of 50 mg/kg of body weight into the gluteal muscle of the left limb. Half of the dose was given on day 0 of the experiment. The second half of the substance was administered to these calves into the gluteal muscle of the right limb on day 4 of the experiment. The third calf received aqua pro injectione, and the fourth animal was given no solution at all.

Determination of T and B lymphocytes. T lymphocytes were detected by the E-rosette method according to Paul et al. (1979). Lymphocytes resuspended in the minimal essential medium (MEM) with 5% fetal calf serum at a concentration of 10^6 in 100 µl (Institute of Sera and Vaccines, Prague) were added to the same volume of 1% suspension of sheep erythrocytes treated with 2-aminoethylisothiouroniumbromid (AET, Serva, Heidelberg).

B lymphocytes were detected by the EAC rosette method described by Komárek (1979). One hundred µl of lymphocytes resuspended in MEM at a concentration of 10^6 were mixed with an equal volume of the erythrocytes-amboceptor-complement complex (Institute of Sera and Vaccines, Prague). The E and EAC rosettes (200 cells) were evaluated using a light microscope (Poland). Rosettes were evaluated on day 0, 4, 7 and 14 of the experiment.

Separation and immunolabelling of lymphocytes for scanning electron microscopy (SEM). Blood was taken from calves no. 1 and no. 3 (Table 1) for SEM. Mononuclear cells (i. e. lymphocytes and monocytes) were separated according to Boyum (1947) and then treated according to the method of De Harven et al.

(1987). The cells were labelled by the indirect immunocytochemical method using colloidal gold. Cells were incubated with IL-A30 monoclonal antibodies against bovine IgM. After rinsing, the cells were incubated with colloidal gold (goat antimurine Ig/GAM-G_{40}, Janssen Pharmaceutica, Beerse, Belgium), fixed, dehydrated and dried at a critical point. After mounting of slides and carbonization, the samples were evaluated by means of SEM at secondary or backscattered electron emission.

Table 1

Morphometry of macrophages in the medulla of lymph nodes in the control and glucan-treated animals

Calf no.	Treatment	Lymph node	Size of macrophages (μm)	Observation period (days)
4	no	lnn. poplitei	$10.06 \pm 0.7824^{+}$	slaughtered on day 0
3	aqua pro injectione	lnn. poplitei	9.92 ± 0.7166	slaughtered on day 7
2	4% glucan	lnn. poplitei	$14.22 \pm 0.8072^{*}$	slaughtered on day 7
		lnn. hepatici	11.18 ± 1.2110	
		lnn. mesenterici	10.98 ± 1.1400	
1	4% glucan	lnn. poplitei	$12.80 \pm 1.2328^{*}$	slaughtered on day 14
		lnn. hepatici	11.12 ± 1.2270	
		lnn. mesenterici	10.76 ± 0.9499	

*Significantly different from animals no. 3 and 4 ($P < 0.01$); +Mean ± standard deviation

Slaughtering of animals. On day 0 of the experiment an untreated control calf, on day 7 a glucan-treated calf and a calf treated with aqua pro injectione, and on day 14 a glucan-treated calf was slaughtered.

Processing of histological material. Samples for histological examination were taken from the right and left popliteal lymph nodes (lnn. poplitei), from the hepatic lymph nodes (lnn. hepatici) and cranial mesenteric lymph nodes (lnn. mesenterici craniales), and from the liver, spleen and lungs. The material was processed by a standard method, i.e. fixed in 10% neutral formalin and embedded in paraffin. The 5–6 μm thick sections were stained with haematoxylin and eosin, by Giemsa staining and by the periodic acid–Schiff reaction (PAS).

Morphometry of phagocytes in lymph nodes. Measurement was performed by the eyepiece and stage micrometer (Meopta, Prague) with a light microscope

(Meopta, Prague). The length and width of phagocytes were measured at a total magnification of × 450 in the medullary sinusoids of lymph nodes and their ratio was determined. One hundred cells were evaluated in each lymph node.

Electron microscopic examination. Small samples from the medulla of lymph nodes from calves of both groups (control and experimental) were immediately immersed in a fixative containing 2.5% glutaraldehyde and 2.0% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.2). Tissues were postfixed in 1% OsO_4, dehydrated in ethanol series, embedded in Durcupan and cut on an LKB Nova ultramicrotome. Semithin sections (1–2 μm) were stained with 1% toluidine blue and examined in a light microscope (Carl Zeiss Jena). Ultrathin sections were mounted on copper grids and stained with uranyl acetate and lead citrate. Sections were examined in a Jeol 1200 MX transmission electron microscope at 80 kV.

Statistical analysis. The statistical significances of the data were evaluated by Student's *t*-test.

Results

No gross lesions were observed at postmortem examination of the animals slaughtered.

Histological examination of the lymph nodes of glucan-treated animals showed changes in the number and size of macrophages present in the medullary and paracortical sinusoids (Figs 1 and 2). The average size of macrophages in medullary sinusoids is presented in Table 1. The size of macrophages in lymph nodes of the glucan-treated animals was significantly different from that in the control calves. Enlarged macrophages in the sinusoids of lymph nodes presented PAS-positive fibrillar structures in the vacuolated cytoplasm.

Electron microscopy revealed that the vacuoles of macrophages were filled with fibrillar component on the periphery. This often stratified and irregularly round structure was seen in the cytoplasm of macrophages. Around these structures a granular component was observed. The vacuole centre was filled with amorphous component (Fig. 3). Some vacuoles were solitary and reached a considerable size, pressing the nucleus to the periphery of the cell, or they were numerous, filled only with amorphous component with a small amount of granular particles along their circumference. Macrophage morphology showed different stages of necrobiosis. No morphological changes were found in the T and B zones of lymph nodes.

Numerous areas with small mononuclear cell infiltration were found in the liver. Infiltration was most often seen in the portobiliary spaces, near the central veins of hepatic lobules. They consisted of freely accumulated cells, predominantly histiocytes and monocytes with some lymphocytes and, sporadically, also

polymorphonuclear leucocytes. Kupffer's cells were packed with phagocytic material. No mononuclear granulomas were observed in the lungs and spleen.

The mean values of T and B lymphocytes in the two groups of animals ranged from 38.0% to 40.1% and from 14.5 to 16.1%, respectively.

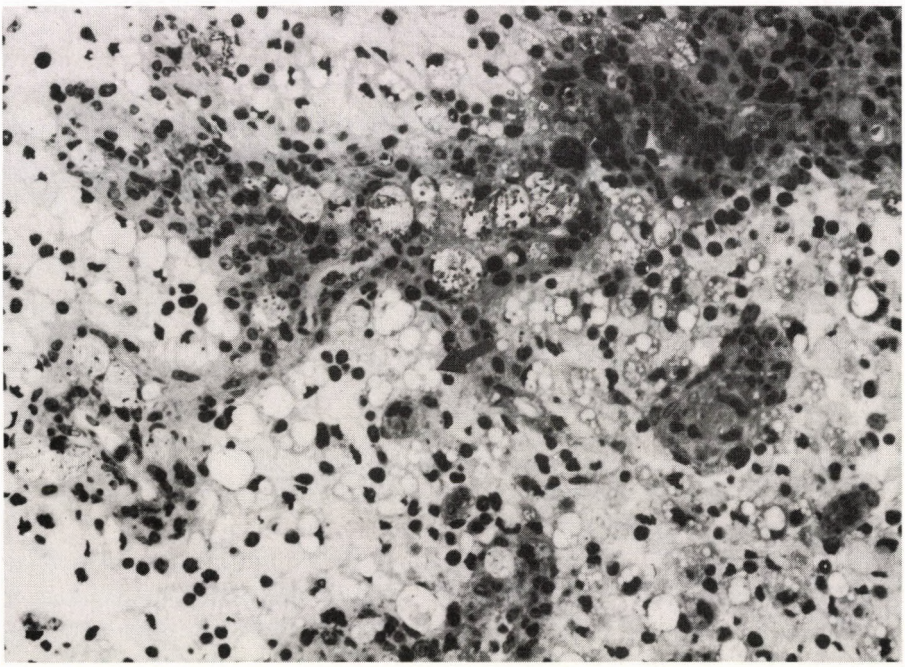


Fig. 1. Enlarged vacuolated macrophages in medullary sinusoids of lymph nodes. A calf on day 14 after glucan administration. Semi-thin section stained with toluidine blue, × 80

The surface of the majority of B lymphocytes (Fig. 4) from the glucan-treated calf slaughtered on day 14 (no. 1) showed an increased expression of IgM molecules (Fig. 5) as compared to the expression of IgM molecules on the surface of B lymphocytes from the control calf (no. 3).

Discussion

Both the macroscopic and microscopic examinations of animals treated with soluble glucan confirmed previous results that this form of glucan does not induce hepatosplenomegaly (Di Luzio, 1985), not even after a dose of 50 mg/kg of body weight.

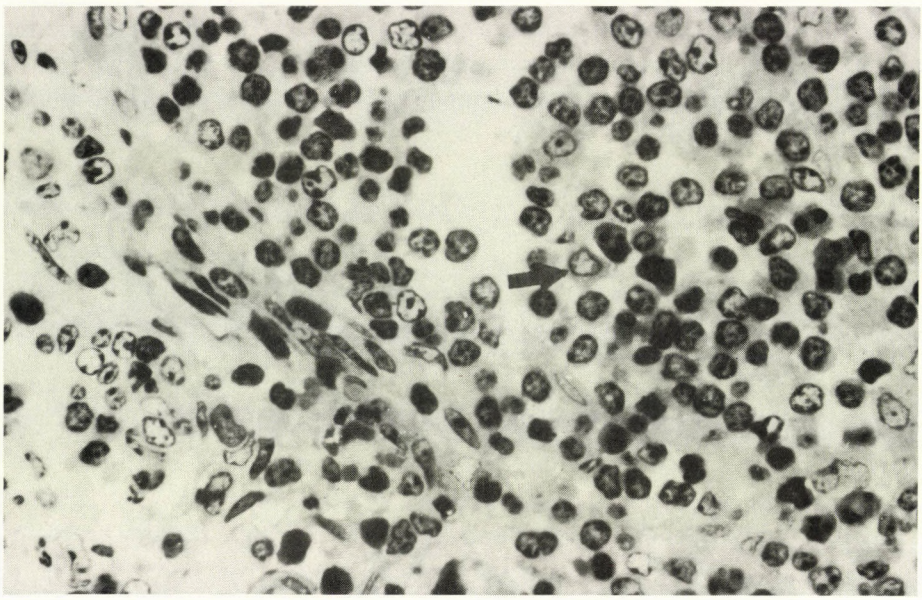


Fig. 2. Macrophages in medullary sinusoids of lymph nodes. A control animal. Semi-thin section stained with toluidine blue, × 80

The histologically demonstrated mononuclear infiltrations and activated Kupffer's cells seen in the liver indicate the mobilisation of macrophages in this organ. Such changes have been observed in animals after application of particulate glucan (Williams et al., 1985). Deimann and Fahimi (1979) found that particulate glucan resulted in the development of mononuclear granulomas in the liver, which made up approx. 10% of total parenchymal volume. They attributed the formation of granulomas to the mobilisation of monocytes from the bone marrow and to the less pronounced proliferation of Kupffer's cells. In the present study, administration of a relatively high dose of soluble glucan was followed by the less extensive presence of mononuclear infiltration, which was manifested also in the preserved size of the liver.

Macrophages in the lymph nodes of glucan-treated animals showed quantitative and significant qualitative differences from those seen in the lymph nodes of control animals. Accumulation and enlargement of macrophages in lymph nodes following exposure to macrophage-activating agents seem to represent an important stage of macrophage activation. A significant degree of macrophage activation was observed both in a regional lymph node and in lymph nodes more distant from the site of glucan application. This indicates a first-rate effect of glucan on cells of the mononuclear phagocyte system of lymph nodes in the entire organism.

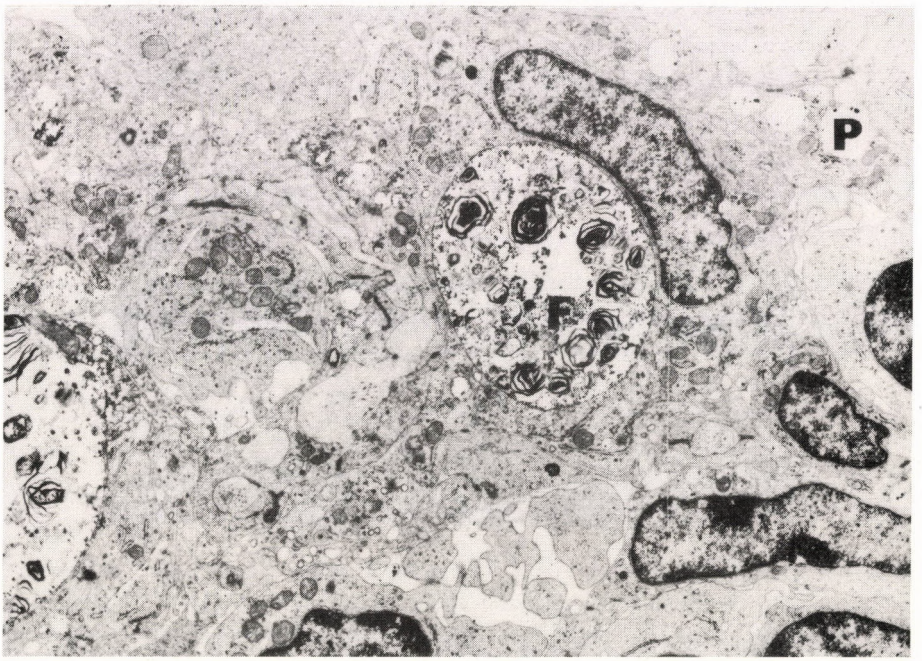


Fig. 3. Vacuoles in the cytoplasm of macrophages with fibrillar, particulate and homogeneous component (F), or with particulate and homogeneous component (P). A calf on day 14 after glucan administration. Electron micrograph, × 3,000

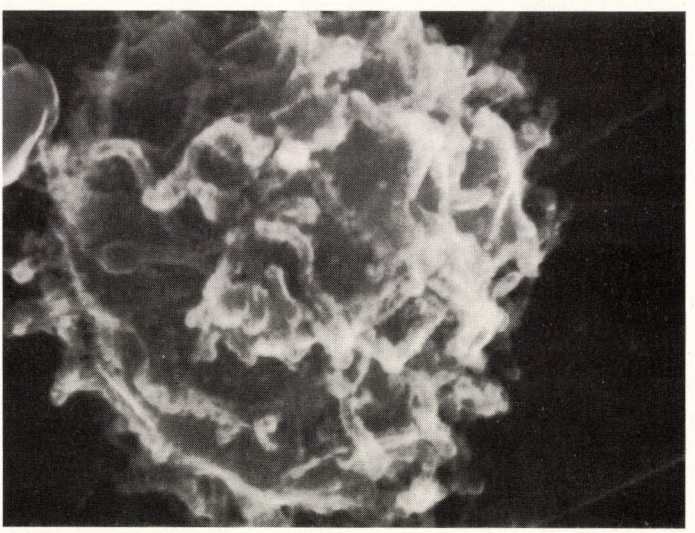


Fig. 4. Secondary electron image. Bovine peripheral B lymphocyte isolated from a calf on day 14 after glucan administration. × 21,000

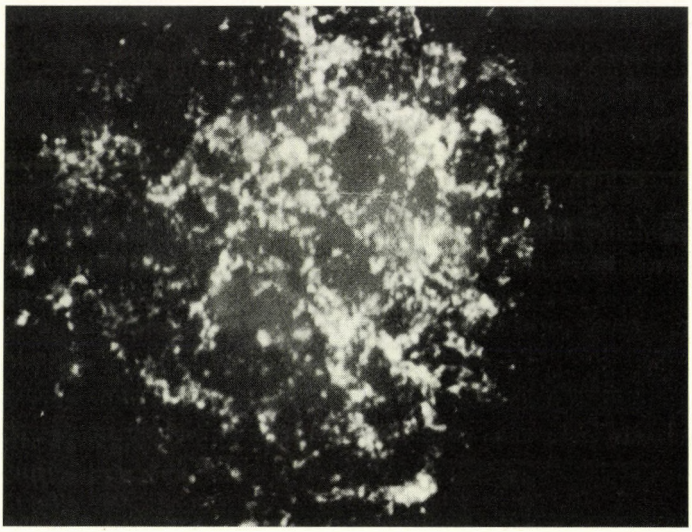


Fig. 5. Backscattered electron image. The same lymphocyte as in Fig. 4. Lymphocyte labelled with 40 nm colloidal gold after incubation with Moab IL-A30 against IgM. A considerable number of colloidal gold particles can be seen on the surface. × 21,000

The smaller diameter of macrophages measured on day 14 compared with that on day 7 after application indicates that by day 14 other mobilised macrophages had appeared in the sinusoids. The presence of also necrotic macrophages on day 14 after application supports this assumption. The increased occurrence of enlarged and necrotic phagocytes in a regional lymph node on the day of slaughter also indicates that the above-mentioned doses of glucan were rather high. What had to be determined was the extent of stimulating or toxic effects exerted by these doses on macrophages.

It is well known that glucan is a powerful activator of mononuclear phagocytic cells (Browder et al., 1987). Macrophages as target cells for glucan have been suggested to be stimulated to higher metabolic activity, and they release a larger amount of interleukin-1 which is an accessory factor of T cell activation (Chihara, 1984). Effects of this substance on the activity of cellular immune response, especially its adjuvant action, are significant (Bousquet et al., 1989; Benda and Mádr, 1991; Benková et al., 1992). However, in the present study no marked changes occurred in the levels of blood T and B lymphocytes.

Expression of immunoglobulins on the surface of B lymphocytes can be studied and quantitated very precisely by immunoscanning electron microscopy (Levkut et al., 1991). The increased expression of IgM molecules on the surface of B lymphocytes from the glucan-treated calf slaughtered on day 14, in compari-

son with the expression of IgM molecules on the surface of B lymphocytes from the control calf slaughtered on day 7, suggests the activation of B cells in a non antigen-specific manner. It seems likely that cytokines played a dominant role in the activation of B cells after glucan administration.

The main conclusions are as follow: (*i*) no macroscopic changes were seen in the organs of calves treated with glucan at a dose of 50 mg/kg of body weight; (*ii*) microscopically visible mononuclear infiltrations were found only in the liver; (*iii*) quantitative and major qualitative changes were demonstrated in the size of macrophages in the lymph nodes. The study of macrophage activation also indicated the speedy removal of the glucan doses administered and the subsequent profound reaction of the mononuclear phagocyte system in lymph nodes. The considerable mobilisation of these cells, seen only in these tissues of the immune system after glucan administration, suggests the possibility of using this substance, even in single high doses, in the treatment of various lymphadenopathies of chronic course, or for supporting the immune system. As was shown by electron microscopy already on day 7 of the experiment, a non-staining fluid, probably representing a degradation product of this polysaccharide, could be seen besides the fibrillar substance (PAS-positive reaction by light microscopy).

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A CASE OF METASTASIZING OVARIAN GRANULOSA CELL TUMOUR IN THE MYOCARDIUM OF A BITCH

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Clinical examinations including X-ray analysis, gross pathological, histopathological and ultrastructural investigations were performed on a case of ovarian granulosa cell tumour in a 5-year-old German boxer bitch. Radiographic examination of the abdomen revealed a round, sharply demarcated formation about 4–5 cm in size. The tumour of the left ovary was of a malignant granulosa cell type, with numerous metastases 5–20 mm in size, present in the myocardium (left ventricle), lungs (in all lobes) and prescapular lymph node. Ultrastructural characterization of the tumour demonstrated closely packed cells, with rough endoplasmic reticulum, indicating markedly enhanced protein synthesis probably associated with rapid cell proliferation.

Key words: Ovary, myocardium, metastasis, bitch

Tumours are very common in the dog and particularly in the bitch. A possible explanation of this could be the relatively long lifespan of dogs and the numerous predisposing factors affecting them, including the urban environment. Among the tumours reported in dogs, mammary tumours are the type predominantly described. In a study involving 400 bitches, neoplasms of the ovaries were detected in 115 animals (28.75%) (Dow, 1960). These tumourous lesions were more common in older animals, and their incidence increased with age. Granulosa cell tumours were found in 13 bitches aged 5 to 15 years, and 10 of these animals did not have litters. The tumours were usually round or oval in shape, and measured 0.4–10 cm in diameter. It should be stressed that no metastases were found in any of these cases. However, in one case, a tumour nodule was found in the other ovary by microscopic examination.

Bostock and Owen (1975) stated that ovarian tumours were the most frequent in mares and bitches. In mares, ovarian tumours originated from Graafian follicle cells which were able to secrete oestrogen and cause permanent oestrus,

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along with the development of pathological changes. According to their report, ovarian tumours of the bitch induced less obvious changes, and were clinically similar to pseudopregnancy with an enlarged abdomen. It was stated that granulosa cell tumours could grow quite large before diagnosis was established.

Naby Cheng (1992) reported that 1.2–1.4% of all primary neoplasms in the bitch were ovarian tumours. Sixteen to 21% of the ovarian tumours were granulosa theca cell tumours. Naby Cheng (1992) also reported that granulosa cell tumours occurred most often in older bitches and that metastases could be observed, with a resulting dysfunction of the affected organ. The clinical findings included alopecia, pyometra, and cystic endometrial neoplasm.

Of 4356 dogs examined post mortem in Piedmont and Lombardy during the past 10 years, 38 had neoplasms of the heart base (17 primary and 21 metastatic), but none of these metastases arose from ovarian tumours. Eleven of the primary neoplasms arose from aortic bodies. Five of the primary neoplasms and four of the metastases were found in Boxers, whereas four of each kind were observed in German Shepherds (Cammarata et al., 1987).

Panciera et al. (1991) reported on two 4-year-old mares, one with simultaneous and the other with successive occurrence of ovarian teratoma and granulosa cell tumours, but found no metastases in the heart.

Janiszewski (1992) described a case of an 11-year-old bitch with a tumour which was found to originate from the left ovary upon exploratory laparotomy. The tumour weighed 1.1 kg and was diagnosed histopathologically as a granulosa cell tumour.

Patnaik and Greenlee (1987) examined 71 primary ovarian tumours in the dog and reported that 12 of the tumours (17%) were of the granulosa cell type. Most granulosa cell tumours were solid, but two were mostly cystic. Four granulosa cell tumours were macrofollicular, having cysts lined with granulosa cells. No metastases were found in the myocardium.

The aim of this case report was to demonstrate whether or not an ovarian granulosa cell tumour produced metastases in the myocardium of a bitch.

Materials and methods

A 5-year-old German Boxer bitch was presented for examination and therapy. The case history revealed that the bitch had had decreased appetite over the previous 10 days and had completely stopped eating in the last four days, although she took small amounts of fluids. The bitch was subjected to clinical examination including radiography. After euthanasia, necropsy was performed to obtain data on gross pathology, and to take samples for histopathology and electron microscopy.

Results

Clinical examination

The animal breathed and moved with difficulty, and had not defecated for three days. Faeces was formed normally and urination was regular. Pale mucous membranes and a whitish-yellowish secretion from both nostrils, as well as inspiratory and expiratory dyspnoea were observed. Auscultation revealed sharp respiratory noise, the heart sounds were muffled, and the pulse was frequent (more than 100 per minute, accompanied with respiratory arrhythmia), but weak. However, no pathological murmurs of the heart were detected.

The abdominal wall was quite tense, with the presence of apparent pain on palpation of the mesogastric and hypogastric regions.

Radiographic examination of the lateral shots of the thoracic cavity showed normal shape of the heart which extended between three intercostal spaces.

Three well-demarcated round formations 20 mm in size were observed in the parenchyma of the lungs, within the area of the apical lobes, cranioventrally in line with the second pair of ribs. In the diaphragmatic lobes, above the caudal vena cava, sharply demarcated formations about 3 cm in diameter were visible. A profile of the abdomen of the dorsal mesogastrium, at the same level as, and close to the third lumbar vertebrae, revealed a sharply demarcated formation of round shape, about 4–5 cm in diameter. The cranial outline of the formation was partly superimposed on the dorsocranial edge of the left kidney. The clinical findings and radiographic pictures indicated abnormal changes in the ovaries and enlarged lymph nodes in the abdominal cavity. The alterations in the lungs were consistent with changes due to metastases.

Gross pathology

Necropsy confirmed tumour of the left ovary with metastases in the myocardium (left ventricle), lungs (in all lobes), and in the left prescapular lymph node. There was also diffuse fatty degeneration and congestion due to passive hyperaemia in the liver, and catarrhal desquamative gastroenteritis. Apart from the tumour of the left ovary, there were no visible changes in other parts of the genital and reproductive tract.

Histopathology

Histopathologically, the left ovary was lobulated. In haematoxylin-eosin stained sections numerous typical round and ovoid cells were seen, which contained dark-staining nuclei and an eosinophilic cytoplasm (Fig. 1). The cells were formed into islands and rosettes by the surrounding connective tissue. These find-

ings confirmed our suspicion of the presence of a granulosa cell tumour of the ovaries, with metastases in the lungs and myocardium, as mentioned earlier (Figs 2 and 3).

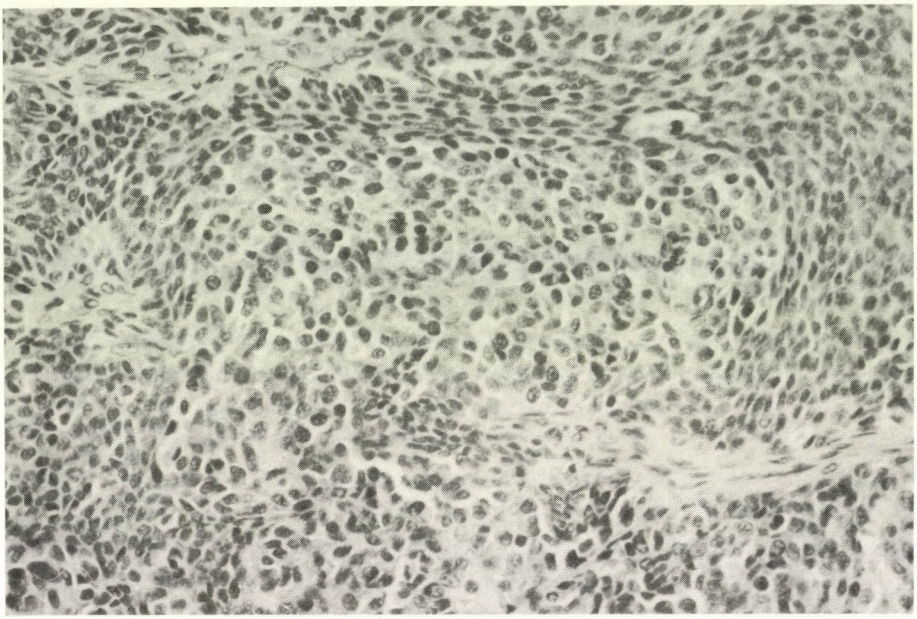


Fig. 1. Granulosa cell tumour formation in the left ovary. Typical round and ovoid cells are seen. Haematoxylin and eosin (HE), × 200

Electron microscopy

To confirm the accuracy of the histopathological findings, a piece of the tumour tissue was examined by electron microscopy. On the upper surface of the cells extensions of the cytoplasm were visible. In the cytoplasm of the cells numerous round and oval mitochondria and extensively developed endoplasmic reticulum with aggregations of ribosomes were observed. Lipid vacuoles and some filamentous areas were also present in these cells. The ultrastructural characteristics of the tumour cells corresponded to the morphology of cells of the granulosa type.

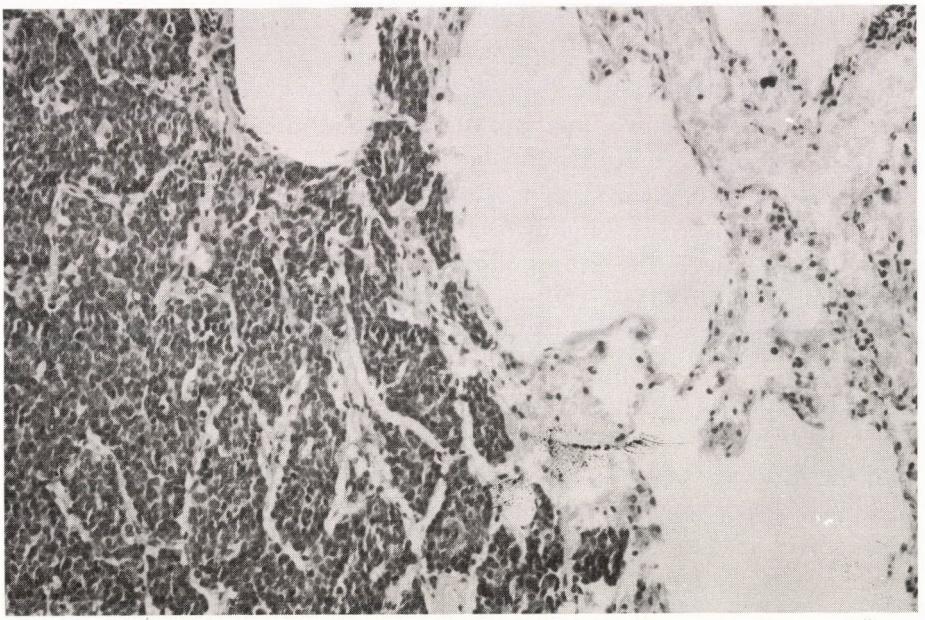


Fig. 2. Metastases of granulosa cell tumour in the lung. HE, × 200



Fig. 3. Metastases of granulosa cell tumour in the myocardium (left ventricle). HE, × 200

Discussion

In bitches, metastatic tumours of the mammary glands are the most common neoplasms. Until now we did not find a case of granulosa cell tumour with confirmed metastases. Early reports by Dow (1960) and Naby Cheng (1992) stated that 16–21% of the ovarian neoplasms consisted of granulosa cell tumours. On the other hand, our case of granulosa cell tumour had typical metastases in the lungs and lymph nodes, as well as in the myocardium of the left ventricle. The latter finding seems to be the first case of metastases of a granulosa cell tumour in the myocardium. These results are at variance with the findings of Dow (1960) who, among 13 confirmed granulosa cell tumours, did not find any case of metastases, only a tumour in the other ovary, by microscopical examination. However, our observations are consistent with the results of Naby Cheng (1992) who reported the incidence of metastases with a resulting dysfunction of the organ affected. Our results point out that metastases in the myocardium caused extreme dysfunction of the heart, as established by clinical examination of the bitch.

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PHARMACOKINETICS OF CEPHALEXIN IN CALVES AFTER INTRAVENOUS AND SUBCUTANEOUS ADMINISTRATION

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The pharmacokinetics of cephalexin was investigated in male calves following a single intravenous (i.v.) and subcutaneous (s.c.) administration (10 mg/kg). The distribution rate constant and distribution half-life were 3.34 ± 1.22 h^{-1} and 0.32 ± 0.08 h, respectively. The values of elimination half-lives following i.v. and s.c. administration were 3.17 ± 1.30 and 2.56 ± 0.24 h, respectively. The absorption half-life following s.c. administration was 0.09 ± 0.01 h. The apparent volume of distribution was 0.65 ± 0.16 and 0.46 ± 0.07 L/kg and total body clearance was 2.96 ± 0.46 and 1.66 ± 0.31 ml/kg/min, respectively, following i.v. and s.c. administration. Systemic availability following subcutaneous administration was found to be almost complete (103.08%). The satisfactory intravenous dosage regimen of cephalexin for calves would be 11.3 mg/kg as the priming dose followed by 10.0 mg/kg as the maintenance dose to be repeated at 6-h intervals. However, the subcutaneous dosage regimen of cephalexin in calves would be 8.0 mg/kg followed by 7.05 mg/kg at 8-h intervals.

Key words: Pharmacokinetics, bioavailability, cephalexin, intravenous, subcutaneous, dosage regimen, calf

Cephalexin, a semisynthetic bactericidal antibiotic, is effective against many Gram-positive and Gram-negative bacteria including the penicillin-resistant strains of *Staphylococcus aureus* (Muggleton et al., 1968). Pharmacokinetic studies of cephalexin administered intravascularly and extravascularly have been conducted in dogs and cats (Silley et al., 1988), cattle (Carli et al., 1983; Soback et al., 1987; Garg et al., 1992) and buffaloes (Garg et al., 1990). However, the disposition of this antibiotic following subcutaneous administration has not been studied in calves. Subcutaneous administration is preferred in some animals be-

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cause of rapid absorption, good availability and ease of administration (Jernigan et al., 1988; Wilson et al., 1989). The aim of the present investigation was to study the pharmacokinetics of cephalexin in male calves following intravenous and subcutaneous administration in comparison with the pharmacokinetic variables obtained after intramuscular administration of the drug.

Materials and methods

Five healthy male calves (73.5 to 93 kg) were used. The animals were fed green fodder and wheat straw, and water was provided *ad libitum.* A freshly prepared solution (10%) of cephalexin (cephalexin monohydrate, Ranbaxy Laboratories Ltd., India) was administered into the jugular vein at a dose of 10 mg/kg body weight. Blood samples were collected into heparinized tubes from the contralateral jugular vein at 5, 10, 15, 30 and 45 min and 1, 1.5, 2, 3, 6, 9 and 12 h after drug administration. Plasma was separated after centrifugation at room temperature. After a washout period of 21 days, cephalexin was administered subcutaneously in the neck region of the same animals. The dose and sampling times were the same as in the intravenous study. The plasma was stored at –20 °C and assayed for cephalexin concentration within 10 days of collection.

The concentration of cephalexin in plasma was determined by a microbial assay method (Arret et al., 1971) using *Sarcina lutea* (ATCC 9344) as test organism. The lower limit of sensitivity of the assay was 0.5 μg/ml.

The plasma cephalexin concentrations of individual calves as a function of time were analysed by curve-stripping (CSTRIP), to determine whether data can best be described by mono-, bi- or triexponential equation, and nonlinear least squares regression (SASNONLIN) computer programmes. After i.v. administration, the disposition of cephalexin followed a two-compartment open model and was described in terms of the biexponential equation

$$Cp = Ae^{-\alpha t} + Be^{-\beta t},$$

where Cp is cephalexin concentration at time t, A and B are zero-time intercepts of initial and terminal phases of plasma concentration-time curves, α and β are the distribution and elimination rate constants, respectively, and e represents the base of natural logarithm. However, following s.c. administration, cephalexin followed a one-compartment open model in terms of the following equations

$$Cp = Be^{-\beta t} - Áe^{-Kat},$$

where Cp is the plasma concentration at time t; Á, B, Ka and β are the values given in Table 2.

After fitting the data to suitable biexponential equations, further pharmacokinetic parameters were calculated by applying the standard equations (Baggot, 1977; Gibaldi and Perrier, 1982). The values of area under the drug plasma concentration curve (AUC) and bioavailability (F) were calculated by following the equation:

$AUC = A/\alpha + B/\beta$ for intravenous route;

$AUC = B/\beta - A'/Ka$ for subcutaneous route.

$$F = \frac{\beta \text{ (mean)} \times \text{AUC (mean) for s.c. route}}{\beta \text{ (mean)} \times \text{AUC (mean) for i.v. route}}$$

Based on the disposition kinetic data, the suitable dosage regimens of cephalexin for calves were calculated (Baggot, 1977).

Results and discussion

The mean plasma concentrations of cephalexin in calves at various time intervals after i.v. and s.c. doses of 10 mg/kg body weight are shown in Table 1. Following i.v. injection, the peak plasma concentration (53.2 ± 2.02 μg/ml) at 5 min declined rapidly during the initial disposition phase. The drug could be detected in the plasma (1.58 ± 0.28 μg/ml) up to 6 h. After s.c. injection (10 mg/kg), absorption of cephalexin into the blood stream was very rapid, as a high concentration (9.10 ± 0.82 μg/ml) of the drug was detected in the plasma as early as 5 min post-injection. The peak plasma concentration (C_{max}; 33.10 ± 1.84 μg/ml) was observed at 45 min (t_{max}) after injection (Table 1). Cephalexin could be detected in the plasma (1.00 ± 0.37 μg/ml) up to 12 h.

The pharmacokinetic parameters describing the distribution, absorption and elimination of cephalexin in calves following i.v. and s.c. administration are presented in Table 2. The high value of distribution rate constant (α; 3.34 ± 1.22 h^{-1}) and the low value of distribution half-life ($t_{½\alpha}$; 0.32 h) indicate that cephalexin is rapidly distributed following i.v. administration. Compared to buffalo calves ($t_{½\alpha}$; 0.13 ± 0.02 h), distribution in bovine calves was slower (Garg et al., 1990).

Following s.c. administration, the high plasma concentration of cephalexin within 5 min and the short absorption half-life (0.09 ± 0.01 h) suggested rapid absorption of the drug. Absorption half-life after s.c. administration in bovine calves was almost comparable to that reported for buffalo calves (0.13 h) following i.m. administration (Garg et al., 1990).

Table 1

Plasma levels of cephalexin in bovine calves following intravenous and subcutaneous administration at a dose rate of 10 mg/kg body weight

Time after administration	Route of administration	
	Intravenous[a]	Subcutaneous[b]
5 min	53.20 ± 2.02	9.10 ± 0.82
10 min	43.30 ± 2.13	13.92 ± 0.53
15 min	35.90 ± 3.43	21.42 ± 2.11
30 min	28.80 ± 3.96	29.25 ± 1.46
45 min	20.00 ± 3.18	33.10 ± 1.84
1 hour	15.10 ± 1.86	24.42 ± 1.90
1.5 hour	10.90 ± 1.53	18.55 ± 1.82
2 hours	7.08 ± 0.95	12.77 ± 1.48
3 hours	3.92 ± 0.38	7.94 ± 1.82
6 hours	1.58 ± 0.28	4.89 ± 1.43
9 hours	N.D.	2.66 ± 1.07
12 hours	N.D.	1.00 ± 0.37

N.D. = Not detected; [a]Values given are mean ± SEM of results obtained for 5 animals; [b]Values given are mean ± SEM of results obtained for 4 animals

The elimination half-lives of cephalexin after intravenous and subcutaneous administration were 3.17 and 2.56 h, respectively, which were similar (2.16–2.43 h) to those reported for buffalo calves following intravenous and intramuscular administration (Garg et al., 1990). The elimination half-life after s.c. administration was slightly longer (2.56 h) than that after i.m. administration (2.0 h) in calves (Garg et al., 1992). However, it was considerably longer than the value (1–1.5 h) reported for calves after i.m. administration (Carli et al., 1983), and the value of 1.4–1.7 h observed after oral, s.c. or i.m. administration in dogs and cats (Silley et al., 1988).

The systemic availability of cephalexin after s.c. administration to calves appeared to be almost complete as the value of F was found to be almost unity (1.03). Data on the bioavailability of cephalexin following s.c. administration in animals is apparently lacking. However, the almost complete and higher systemic availability value of cephalexin (103%) and the comparable or higher values of C_{max} (33.10 ± 1.84 μg/ml) and biological half-life (2.56 ± 0.24 h) following s.c. administration (present study), compared to the values of systemic availability

Table 2

Pharmacokinetic parameters of cephalexin after its single intravenous and single subcutaneous administration at a dose rate of 10 mg/kg body weight to male calves

Pharmakokinetic parameters (Units)	Route of administration	
	Intravenous[a]	Subcutaneous[b]
A (μg/ml)	49.10 ± 3.42	–
α (h^{-1})	3.34 ± 1.22	–
t½ α (h)	0.32 ± 0.08	–
A' (μg/ml)	–	25.08 ± 9.02
Ka (h^{-1})	–	7.96 ± 1.09
t½ Ka (h)	–	0.09 ± 0.01
B (μg/ml)	13.70 ± 5.01	23.24 ± 3.40
β (h^{-1})	0.36 ± 0.09	0.27 ± 0.03
t½ β (h)	3.17 ± 1.30	2.56 ± 0.24
$Vd_{(area)}$ (L/kg)	0.65 ± 0.16	0.46 ± 0.07
Cl_B (ml/kg/min)	2.96 ± 0.46	1.66 ± 0.31
AUC (μg.h/ml)	61.70 ± 9.50	84.80 ± 18.66
Obs C_{max} (μg/ml)	–	33.10 ± 1.84
t_{max} (h)	–	0.75 ± 0.00
F (%)	–	103.08

[a]Values presented are mean ± SEM of results obtained for 5 animals; [b]Values presented are mean ± SEM of results obtained for 4 animals;

A – intercept for distribution phase;
α – overall distribution rate constant;
t½ α – distribution half-life;
A' – intercept for absorption phase;
Ka – absorption rate constant;
t½ Ka – absorption half-life;
B – intercept for the elimination phase;
β – elimination rate constant;
t½ β – elimination half-life;
$Vd_{(area)}$ – volume of distribution;
Cl_B – total body clearance of drug;
AUC – area under the plasma concentration-time curve;
Obs C_{max} – observed peak plasma concentration;
t_{max} – time of peak plasma concentration;
F – bioavailability.

(73.9–81.9%), C_{max} (15.6–32.5 μg/ml) and t½ β (1.49–2.00 h) obtained after i.m. administration (Carli et al., 1983; Garg et al., 1992) to calves, suggest that the s.c. route should be preferred over the i.m. route due to ease of administration, equal rapidity of absorption (t_{max} : 45 min) and lower risk of damage to the intramuscular structures.

The minimum inhibitory concentrations for most of the cephalexin-sensitive bacteria isolated from cattle, dogs, cats and pigs have been reported to be 1.4–4.0 μg/ml (Soback et al., 1987; Silley and Brewster, 1988; Silley et al., 1988). *In vitro* studies of Silley and Brewster (1988) have revealed that a peak concentration of twice the MIC of cephalexin is sufficient to exert the bactericidal effect and that peak antibiotic concentration is of prime importance in determining the efficacy of the drug. Most of the invading bacteria are likely to be killed at a plasma concentration of 2 μg/ml. Therefore, based on the pharmacokinetic data, the suitable dosage regimen was calculated by the formula:

$$D' = Cp\ (min)^{\infty} \times V_{d(area)}^{(e\beta\tau-1)},$$

where D' is the maintenance dose, Cp $(min)^{\infty}$ is the MIC (2 μg/ml); $V_{d(area)}$ and β values were used from Table 2 and τ is the dosage interval. The priming dose (D) is obtained by omitting −1 from the above equation. The priming and maintenance doses by the i.v. route should be 11.3 and 10.0 mg/kg body weight, respectively, to be repeated at 6-h intervals. For the s.c. route, the priming dose should be 8.00 mg/kg and required to be repeated at 8-h intervals at a dose of 7.05 mg/kg body weight.

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ONTOGENY OF THE GENERATION OF DIIODOTHYRONINES (3,3'-T_2 AND 3',5'-T_2) FROM TRIIODOTHYRONINES IN THE LIVER AND KIDNEY DURING THE FETAL LIFE OF THE PIG

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The present study was conducted to obtain information on the generation of diiodothyronines from triiodothyronines (3,3'-T_2 from T_3, and rT_3, and 3',5'-T_2 from rT_3) as a result of the activity of the tissue monodeiodinase enzymes (MD) in the liver and kidney of pig fetuses and their mothers between 32 and 113 days of gestation. T_3-5-MD activity in the fetal kidney during the gestational period was stable and higher than in the liver and in the maternal kidney. In contrast, T_3-5-MD activity of the liver was 3–4 times lower in fetal than in maternal tissue in the first half of pregnancy, and in the second half of pregnancy 3,3'-T_2 production from T_3 in the maternal liver was equal to, or lower than, that in the fetal liver. The activity of MD deiodinating rT_3 (3,3'-T_2 and 3',5'-T_2 generation) increased significantly in fetal liver and kidney in the last 2–3 weeks of pregnancy and was higher than in maternal tissues. In both tissues examined the inner ring deiodinating activity (IRD) was 5–10 times lower as compared to the outer ring (ORD).

Key words: Fetal pigs, triiodothyronines, diiodothyronines, monodeiodinases, deiodination, liver, kidney

The generation of triiodothyronines and diiodothyronines in the peripheral tissues is the result of serial monodeiodination processes. The monodeiodinations affect the outer ring deiodination (ORD) or inner ring deiodination (IRD) of the iodothyronine molecule in a sequential manner and are controlled by specific enzyme systems (Kohrle, 1994). The systems include three different enzymes. The first, 5'-monodeiodinase (5'MD) of type I, is present predominantly in the liver, kidney and thyroid; this is a transmembrane selenoenzyme possessing both ORD and IRD activity (Berry and Larsen, 1992; Kohrle, 1994) and is important for the peripheral production of T_3 from T_4. The second, 5'MD of type II, is present in

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the pituitary, brain and brown adipose tissue (BAT) (Visser, 1988), shows only ORD activity, and is important for local or intracellular T_3 production. The third, 5-monodeiodinase of type III, is found in the central nervous system (CNS), placenta and skin (Leonard and Visser, 1986; Visser, 1990), possesses only IRD activity and is responsible for the degradation of thyroxine (T_4) and 3,3',5-triiodothyronine (T_3).

Thyroid hormone metabolism, including the monodeiodination of triiodothyronines, has been particularly extensively studied on adult animals (Kaplan et al., 1979; Hufner and Grussendorf, 1980; Leonard and Rosenberg, 1980; Kohrle et al., 1987; Visser et al., 1988). In the fetal tissues, 5'-MD-I activity has been detected in the liver and kidney of sheep (Wu et al., 1986*a*; Polk et al., 1988), rats (Galton et al., 1991; Obregon et al., 1991; Ruiz de Ona et al., 1991) and rabbits (Segall-Blank et al., 1981; Brzezińska-Ślebodzińska and Krysin, 1990); 5'-MD-II activity in the pituitary, brain and CNS of rats (McCann et al., 1984), sheep (Polk et al., 1988) and in the BAT of rats (Leonard et al., 1982; McCann et al., 1984; Obregon et al., 1989), rabbits (Brzezińska-Ślebodzińska and Krysin, 1990; Brzezińska-Ślebodzińska and Kapluk, 1990), and sheep (Wu et al., 1986*b*). The 5-MD-III has been studied in the CNS, brain (McCann et al., 1984; Huang et al., 1986), liver and kidney of rats (Huang et al., 1988), and in the liver, kidney and BAT of rabbits (Brzezińska-Ślebodzińska and Krysin, 1990).

Most recently both the 5'- and the 5-monodeiodinations of thyroxine have been studied in the fetal pig (Ślebodziński and Brzezińska-Ślebodzińska, 1994). This paper is the first to show the further step in the sequential monodeiodination of thyroxine, i.e. the deiodination of triiodothyronines to diiodothyronines (3,3'-T_2 and 3',5'-T_2) in the liver and the kidney of pigs at different stages of fetal life.

Materials and methods

Animals. Pig fetuses (n = 67) from 10 litters obtained by Caesarian section between days 32 and 113 of gestation were used in the experiments. Liver and kidney samples from the fetuses and their mothers were quickly dissected out and chilled on ice. The monodeiodination of triiodothyronines (T_3 and rT_3) was measured in the fetal pig from day 32 (in the liver) or 46 (in the kidney) of gestation until term.

Reagents. 3-T_1, 3'-T_1, 3,3',5-T_3, and 3,3',5'-rT_3, were purchased from Henning, Berlin GmbH; $Na^{125}I$ (specific activity 15.7 mCi/mg) from Amersham, England; Chloramine-T from BDH Limited Poole, England; Sodium Pyrosulphite from Merck, Germany; Dithiothreitol (DTT) from Sigma Chemical Co., St. Louis,

MO, USA; 8-anilino-1-naphthalene-sulphonic acid (ANS) from K & K Lab. Inc., Plainview, NY, USA.

Homogenate preparation. The liver and kidney samples were homogenized in 3 volumes (w/v) of ice-cold 0.05 M Tris-HCl buffer (pH 7.2) containing 0.25 M sucrose and 0.005 M EDTA. The homogenate was centrifuged at 10,000 × *g* for 30 min at 4 °C and the supernatant was used immediately for the T_3 and rT_3 deiodination assays.

T_3 and rT_3 monodeiodination in vitro. The activities of T_3-5-MD, rT_3-5'-MD and rT_3-3-MD were determined in the liver and kidney homogenates as described previously (Nowak, 1987). In short, 0.05 ml liver and kidney supernatants were incubated in the presence of 0.23 mM T_3 (conversion T_3 to 3,3'-T_2) or 0.06 mM rT_3 (conversion rT_3 to 3,3'-T_2 and to 3',5'-T_2) with 2 mM DTT in 0.05 M Tris-HCl buffer (pH 7.2) for 15 min at 37 °C. After 10 min of incubation 0.2 mg/ml of ANS was added as an inhibitor of binding proteins. The final volume of the incubation mixture was 0.5 ml. The reaction was stopped with 2 volumes of ice-cold 95 % ethanol. The samples were stored at –20 °C and centrifuged before analysis.

The protein concentration, which ranged from 0.5 to 3 mg/ml, was determined by a modification of the method of Lowry et al. as described by Hartree (1972).

Radioimmunoassay. The diiodothyronines (3,3'-T_2 and 3',5'-T_2) generated during incubation were measured in the ethanol extracts by RIA (Laurberg, 1978) and expressed as pmol of iodothyronines generated per mg protein per 15 min. Each sample was determined in triplicate. The 3,3'-T_2 [^{125}I] and 3',5'-T_2 [^{125}I] were prepared according to the method of Weeke and Orskov (1973). The cross-reactivities of the antibodies (the generous gift of Dr. Peter Laurberg) were: $T_4 < 0.001$, $T_3 < 0.29$, $rT_3 < 0.001$ for anti-3,3'-T_2 and $T_4 < 0.00005$, $rT_3 < 0.013$, $T_3 < 0.000003$, 3,3'-$T_2 < 0.000005$ for anti-3',5'-T_2.

Statistical analysis. Student's *t*-test, one-factor analysis of variance and Duncan's new multiple range test were applied. Statistical significance was defined as $P < 0.05$. Values were given as means ± SEM.

Results

Data on the T_3 to 3,3'-T_2 converting activity in the liver and kidney tissues are summarised in Fig. 1. The 5-monodeiodination of T_3 by fetal liver homogenates remained unchanged (0.06 ± 0.007 pmol 3,3'-T_2/mg protein/15', n = 26) between days 39 and 76 of gestation and increased 3-fold ($P < 0.01$) by day 85 reaching the maternal value (0.23 ± 0.024 pmol 3,3'-T_2/mg protein/15', n = 9),

(Fig. 1a). Near term the activity increased significantly ($P < 0.01$), again reaching the maximal values 0.39 and 0.61 ± 0.025 pmol 3,3'-T_2/mg protein/15', n = 9, in maternal and fetal liver, respectively. This activity was 17 times higher than in the first weeks of pregnancy. Contrary to the fetal liver, T_3 to 3,3'-T_2 deiodination activity of the fetal kidney was higher and relatively stable (0.54 ± 0.046, pmol 3,3'-T_2/mg protein/15', n = 46) during the period of observation (Fig. 1b). The twofold increase of enzymatic activity was observed only between days 46 and 56. The inner ring deiodination of T_3 by fetal kidney homogenates was higher than in the maternal tissues.

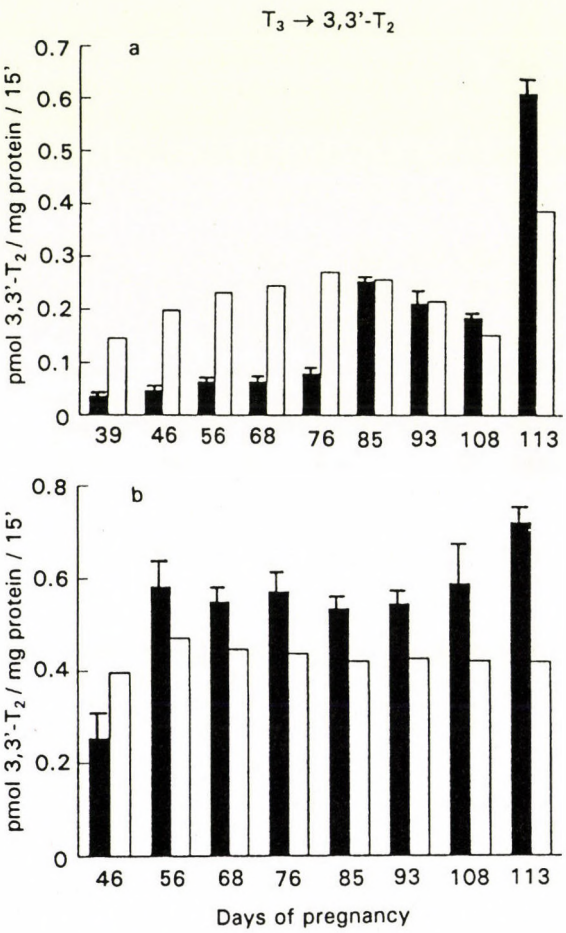


Fig. 1. Changes in T_3 to 3,3'-T_2 conversion of fetal pigs' (solid bars) and mothers' (empty bars) liver (a) and kidney (b) homogenates throughout pregnancy. Values are means ± SEM; n = 5–9

The outer ring deiodination of rT_3 to 3,3'-T_2 (5'-MD activity) did not change in the fetal and maternal liver between days 32 and 68 of gestation (0.96 ± 0.049; n = 25 and 1.49 ± 0.085; n = 5 pmol 3,3'-T_2/mg protein/15', respectively); however, on day 76 the enzyme activity rose about threefold (Fig. 2a), reaching a maximal value on day 108 (6.58 ± 0.34 pmol 3,3'-T_2/mg protein/15', n = 10). The 5'-MD activity in the fetal kidney was stable and low (1.69 ± 0.286 pmol 3,3'-T_2/mg protein/15', n = 27) during three months of pregnancy (between days 46 and 85), while 3 weeks before delivery it increased 4–6 times ($P < 0.01$) (Fig 2b). In the maternal kidney the 5'-MD activity was unchanged until day 108 of gestation (1.47 ± 0.097 pmol 3,3'-T_2/mg protein/15', n = 7) and it was generally lower than in the fetal tissue. As shown in Fig. 2b, one day before delivery it increased about 3-fold reaching the fetal value (5.12 ± 0.062 pmol 3,3'-T_2/mg protein/15', n = 8).

The inner ring deiodination of rT_3 to 3',5'-T_2 (3-MD activity) decreased significantly ($P < 0.05$) in the fetal and maternal liver on day 76 of gestation (Fig. 2c) and in the maternal liver it was unchanged from day 85 until delivery. In the fetal liver the activity increased two- to threefold one week before birth, reaching a maximal value (0.85 ± 0.059 pmol 3',5'-T_2/mg protein/15', n = 9) on day 113. The 3-MD activity in the maternal kidney was relatively stable (0.91 ± 0.017 pmol 3',5'-T_2/mg protein/15', n = 8) throughout pregnancy, contrary to the fetal tissue where it increased at midgestation (between days 56 and 76) (Fig. 2d). One day before delivery the 3-MD activity reached a maximal value (1.36 ± 0.089 pmol 3',5'-T_2/mg protein/15', n = 8) in the fetal kidney and was significantly higher ($P < 0.01$) in relation to that in the maternal tissue.

In general, the inner ring deiodinating activity (T_3 to 3,3'-T_2 and rT_3 to 3',5'-T_2) was 5–10 times lower than the outer ring deiodinating activity (rT_3 to 3,3'-T_2) in both tissues.

Discussion

The present paper demonstrates the generation of diiodothyronines (3,3'-T_2 and 3',5'-T_2) as a result of outer (5') and inner (3 or 5) ring monodeiodination of triiodothyronines (T_3 and rT_3). These studies comprised the fetal period from day 32 of gestation to the day of birth and, to our knowledge, are the first to show the ontogeny of the conversion of triiodothyronines to diiodothyronines in fetal pig tissues.

The activity of the triiodothyronine deiodinase system during the early stages of embryogenesis has been studied especially in birds. It has been described in 5-day-old chick embryos, in the liver and brain of 11-day-old chick embryos

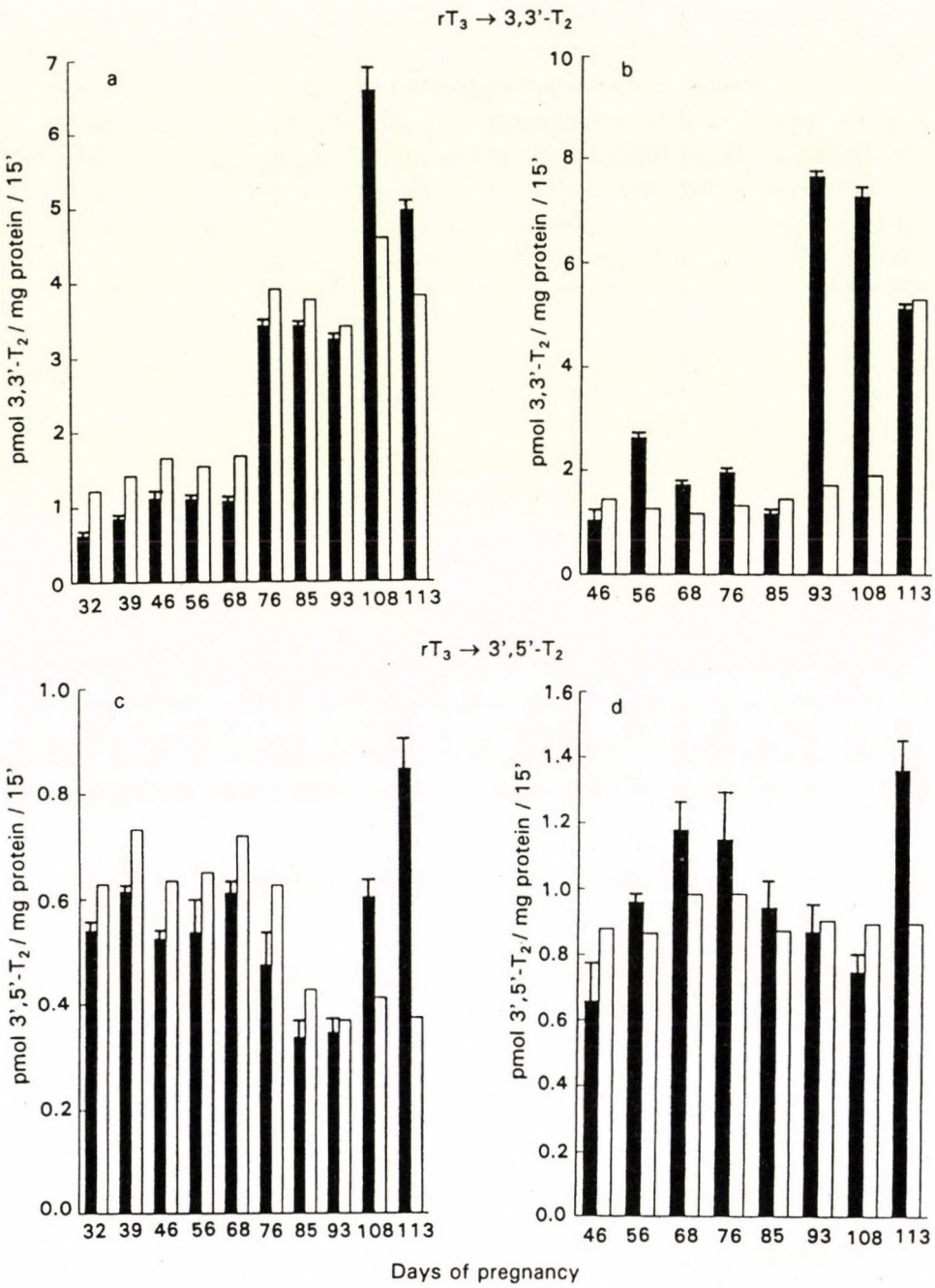


Fig. 2. Changes in rT_3 to 3,3'-T_2 and 3',5'-T_2 conversion of fetal pigs' (solid bars) and mothers' (empty bars) liver (a, c) and kidney (b, d) homogenates throughout pregnancy. Values are means ± SEM; n = 5–9

(Valverde et al., 1993), and in the liver of 12- and 16-day-old Japanese quail embryos (Freeman and McNabb, 1991).

In the present study, iodothyronine monodeiodinase activities rT_3-5'-MD, T_3-5-MD and rT_3-3-MD were found in the fetal liver and kidney from the beginning of the observation period, i.e. already before the pig thyroid became functional (thyroid activity in pigs begins to function at midgestation). Rankin (1941) found that on day 52 of gestation T_4 iodine was present in thyroid tissue. Others (Ślebodziński and Brzezińska-Ślebodzińska, 1994) revealed that quantities of thyroid hormones in the fetal thyroid of pigs can be detected already at day 46 of gestation.

These present findings are consistent with an earlier observation from our laboratory (Ślebodziński and Brzezińska-Ślebodzińska, 1994) according to which thyroxine ORD and IRD system was detected in the fetal pig liver and kidney before the onset of hormonal activity of the fetal thyroid gland. The presence of iodothyronines and active monodeiodinases in the fetal liver and kidney before the fetal thyroid becomes functional suggests that these organs are important sites of enzymatic degradation of maternal iodothyronines during the very early stages of embryogenesis.

Our studies demonstrate that T_3-5-MD activity was 2–3 times higher in the fetal kidney than in the liver, and showed similarity to the observation of thyroxine 5-MD activity in pig fetuses up to day 84 of age (Ślebodziński and Brzezińska-Ślebodzińska, 1994). Contrary to the liver, the fetal kidney (from day 56) was characterized by a higher triiodothyronine monodeiodination activity than the maternal tissues, which coincided with the beginning of fetal thyroid function. It is interesting to note that 3,3'-T_2 generation from T_3 (5-MD) by the liver homogenates was markedly decreased in the fetuses and reached maternal values on day 85 of gestation like in the case of thyroxine 5- and 5'-monodeiodination (Ślebodziński and Brzezińska-Ślebodzińska, 1994). A marked increase was observed in the conversion of rT_3 to 3,3'-T_2 on day 76 of gestation in the fetal and maternal liver and on day 93 in the fetal kidney. One week before birth the rT_3-5'-MD activity was significantly ($P < 0.05$) higher in the fetal than in the maternal tissues.

The sudden increase of ORD and IRD in the fetal liver and kidney before birth was probably relevant to the maturation of the deiodinase systems. It is interesting to note that 5'-MD activity was higher in comparison to 5-MD and 3-MD activity in the tissues examined.

The study performed on fetal and adult rats showed that the ORD activity of rT_3 in the liver was much lower in the fetuses in comparison with adult rats during the last days of pregnancy (Harris et al., 1978; Harris et al., 1979; Cheron et al., 1980; Galton et al., 1991; Ruiz de Ona et al., 1991). This is contrary to our findings in the pig (Ślebodziński and Brzezińska-Ślebodzińska, 1994) which

showed that 5'-MD activity of the liver was higher in the fetal pig than in maternal tissues. These data suggest the importance of the monodeiodination of thyroid hormones for metabolism during the fetal development of the pig.

Recently it has been shown that the monodeiodination of iodothyronines in the liver and kidney is greatly facilitated by sulfation of the phenolic hydroxyl group. It appears that the function of sulfation is to induce the inactivation of the metabolism of thyroid hormones (Visser et al., 1990; Chopra et al., 1992). It has also been demonstrated that T_4S, T_3S and rT_3S are the predominant metabolites of thyroid hormones in the developing ovine fetus (Wu et al., 1993; Polk et al., 1994). Other authors reported that T_3S may serve as a metabolite of T_3 for rapid deiodination or that T_3S is a local source of T_3 in tissues of the rat fetus with low T_3 syndrome (LoPresti et al., 1991; Santini et al., 1992). Sulfation and subsequent deiodination appear to be the significant metabolic pathway for T_4, T_3 and lower iodothyronines in developing mammals but till now this has not been described in the fetal pig.

The present studies are in agreement with earlier results and confirm that the enzymatic system responsible for the degradation of iodothyronines in a cascade fashion (Visser, 1988) is active in the fetal pig already before the onset of thyroid function (Ślebodziński and Brzezińska-Ślebodzińska, 1994; Krysin, 1995; Brzezińska-Ślebodzińska et al., 1995).

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EFFECT OF RETINYL ACETATE, ASCORBIC ACID AND TOCOPHEROL SUPPLEMENTATION OF THE FEED ON EGG VITAMIN A CONTENT IN JAPANESE QUAIL

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The ration fed to laying Japanese quails was supplemented either with retinyl acetate (RA) (50×10^3 IU/kg, group A), ascorbic acid (500 mg/kg, group C), or with both substances in combination with each other (group AC) and with tocopheryl acetate (37.8 IU/kg; groups AE and ACE). On days 1, 8, 14, 20 and 28, some quantitative parameters of eggs (mass of egg-shell, albumen and egg yolk, retinoid content of egg yolk) were measured. The egg production parameters were not significantly affected by the supplementations. By the end of the second week, the total vitamin A (retinyl esters + retinol) concentrations of the egg yolk were significantly higher in the groups receiving supplemented feed (AC, AE and AEC) than in the control group. Two weeks later (on day 28), the vitamin A levels were elevated significantly in all groups except the group treated with ascorbic acid. From the point of view of vitamin A fortification of the egg yolk, the combined supplementations (groups AC, AE, AEC) seem to be more effective. The results indicate that vitamin A content of the egg yolk can be increased by a short-term RA supplementation of the laying ration. The retinoids present in the natural substances of eggs could possibly be a good source of vitamin A for humans.

Key words: Retinol, retinyl esters, tocopherol, ascorbic acid, egg, egg yolk fortification, Japanese quail

The poultry egg represents a potential new chicken as well as a food of high biological value. Its biological value depends on the composition, which is well known in numerous species. The percentage composition (protein, carbohydrate, fat and mineral content) of different parts of the egg (egg-shell, albumen and yolk) is very similar in different species (Sturkie, 1976; Burke, 1984; King and McLelland, 1984). The egg is one of the most valuable foods available to humans: it contains high-quality proteins, high amounts of valuable minerals and vitamins, and has low calorie (energy) content. In addition to the main nutrients (proteins, carbohydrates and fats), the amount of different micronutrients determines the biological value of eggs. For this reason, many experiments have been conducted

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to increase the micronutrient content of eggs. It was found that the polyunsaturated fatty acid (PUFA) content of eggs increased after the addition of small amounts of edible oils to a standard diet (Editorial, 1995). Fortification of eggs with cobalamin was easily accomplished by increasing the vitamin B_{12} content of the diet (Naber and Squires, 1993). In view of the numerous health benefits associated with the consumption of antioxidants such as α-tocopherol and β-carotene, dietary levels necessary for significantly increasing their concentration in chicken egg yolk were determined (Jiang et al., 1994). The consumption of a feed containing selected nature-identical carotenoids enhanced the colour of egg yolk to the desirable level (Latscha, 1990). Carotene fortification is an important factor in increasing the marketability of eggs; however, it may also exert disease-preventing effects based upon the antimutagenic and anticarcinogenic activity (Van Poppel, 1992) and immunomodulatory properties (Bendich, 1992) of some carotenoids.

The aim of this experiment was to study the efficiency of vitamin A transfer from diet — supplemented with retinyl acetate — to egg. Some retinoids participate in oxidation-reduction systems of the organism (Kartha and Krishnamurthy, 1977). For this reason, in addition to retinoid supplementation a well-known water-soluble (ascorbic acid) and a fat-soluble (α-tocopherol) compound and their combination were used as dietary factors in the present experiment.

Materials and methods

Experimental animals and design

Six-week-old laying Japanese quails of a conventional breed were kept in batteries, under natural light. They were fed a commercial laying fodder *ad libitum* for one month. Drinking water was always available. The quails were assigned into groups (n = 10) according to the feed supplementations (Table 1). The eggs laid were collected daily. On day 1, 8, 14, 20 and 28 of the experiment, some quantitative parameters of eggs (mass of egg-shell, albumen and yolk) were measured. On day 1, 14 and 28, five eggs per group were subjected to vitamin analyses. Retinoid determinations were done by HPLC methods (Biesalski et al., 1986), while the quantity of ascorbic acid was measured by colorimetry (Henniger, 1981).

Vitamin preparations used for the experiments

The basal food was supplemented with retinyl acetate using vitamin A capsules 50,000 IU (EGIS, Budapest, Hungary) commonly available in pharmacies. The active ingredient of that formula is retinyl acetate (RA) (Hoffman-La Roche,

Basle, Switzerland). Vitamins A and E were added to the drinking water as a water-miscible formula (Jolovit, BCR, Bábolna, Hungary) also containing RA and DL-α-tocopheryl acetate (TA). The vitamin C supplementations were made by adding L-ascorbic acid (Reanal, Budapest, Hungary) to either the feed or the drinking water.

Statistics

The results are presented as means ± standard deviation (x ± S.D.). The weighted means were used for the evaluation of the egg parameters measured. Statistical comparisons were performed using Student's *t*-test. A level of $p \leq 0.05$ was accepted as significant.

Results

At the beginning of the experiment the vitamin A concentrations of the egg yolk were in the range of 3-5 μg/g. At the end of the 2nd week, the total vitamin A (retinyl esters+retinol) concentrations of the yolk were significantly higher in the supplemented groups (AC, AE and AEC) than in the control. Two weeks later (on day 28), the vitamin A levels were elevated in all groups except in group C receiving ascorbic acid supplementation (Table 2).

In the whole egg homogenate low concentrations of vitamin C (ranging from 11.1 to 14.5 μg/g) were detected in the control and RA-treated groups (C, AC and AEC).

During the experiment, the 60 laying quails of the 6 groups laid a total of 1120 eggs. The arithmetic mean of this value is 186. The number of eggs laid was the highest in group AEC and the lowest in group C. These data are also presented as daily egg-laying performance of the different groups (Table 3).

The values of whole egg mass (about 8 g) and egg-yolk mass (about 2.5 g) were in a similar range in all groups (Table 3).

Table 1

Experimental groups and vitamin A, C and E supplementations

Group	Control	A	C	AC		AE		AEC		
				Basal feed*						
Vitamin supplement	–	RA	AA	RA	AA	RA	TA	RA	TA	AA
Dose	–	50×10^3 IU	500 mg	50×10^3 IU	500mg	50.4×10^3 IU	37.8 IU	50.4×10^3 IU	37.8 IU	500mg
Route of application	–	feed	feed	feed		drinking water		drinking water		
No. of animals	10	10	10	10		10		10		

*containing 8100 IU vitamin A and 9.63 IU vitamin E per kg of feed
AA: L-ascorbic acid; RA: retinyl acetate; TA: DL-α-tocopheryl acetate

Table 2

Vitamin A content (μg/g) of egg yolk

Day of sampling	Groups					
	Control	A	C	AC	AE	AEC
1	3.74 ± 0.55	3.23 ± 0.66	2.98 ± 0.35	5.28 ± 1.57	3.95 ± 0.76	2.92 ± 0.14
14	4.56 ± 0.43	7.42 ± 3.61	4.26 ± 0.24	10.22 ± 3.12*	15.06 ± 6.31*	17.83 ± 1.58**
28	5.49 ± 0.26	13.51 ± 0.66**	4.77 ± 0.96	14.13 ± 1.07*	24.73 ± 1.89***	32.75 ± 8.76**

Values of significance compared to the control group: *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$

Table 3

Egg-laying performance, egg mass and vitamin A concentrations

		Groups					
		Control	A	C	AC	AE	AEC
Total no. of eggs laid[a]		207	185	144	200	172	212
Eggs/day		7.13 ± 2.19	6.37 ± 1.67	4.96 ± 1.34	6.89 ± 1.87	5.93 ± 2.01	7.31 ± 1.87
Egg mass (g)	Whole egg	8.32 ± 0.33	8.91 ± 0.27	7.68 ± 0.12	8.65 ± 0.31	8.28 ± 0.59	8.40 ± 0.42
	Egg-shell	0.83 ± 0.03	0.85 ± 0.04	0.79 ± 0.04	0.86 ± 0.03	0.81 ± 0.03	0.83 ± 0.02
	Albumen	4.78 ± 0.21	4.89 ± 0.24	4.18 ± 0.11	4.65 ± 0.17	4.70 ± 0.48	4.71 ± 0.30
	Yolk	2.67 ± 0.07	2.88 ± 0.17	2.38 ± 0.14	2.88 ± 0.08	2.56 ± 0.08	2.67 ± 0.21
Yolk/albumen		0.55	0.58	0.56	0.61	0.54	0.56
Albumen/yolk		1.79	1.69	1.75	1.61	1.83	1.76
Vitamin A concentration (μg/g) of egg yolk[b]		5.02 ± 0.65	10.46 ± 4.3	4.51 ± 0.36	12.17 ± 2.76*	19.89 ± 6.83**	25.29 ± 10.55**

[a] total number of eggs laid during the experiment; [b] mean of samples taken on days 14 and 28; Values of significance compared to the control group: *$p \leq 0.05$; **$p \leq 0.01$

Discussion

Egg production, egg weight and egg quality were studied in 6- to 50-week-old Japanese quails. The diet was supplemented with retinol added at a dose of 2000, 4000, 6000 and 8000 IU/kg. The egg production, egg weight, albumen weight, egg-yolk weight, egg-shell thickness, and yolk/albumen ratio of egg-line quails were significantly influenced ($p \leq 0.01$) by retinol intake. Albumen weight, egg-shell weight, egg-shell thickness, and the measured egg quality traits improved with increasing retinol intake up to 6000 IU/kg. It was concluded that the optimum dose of vitamin A supplementation was 6000 and 4000 IU/kg for egg- and meat-line Japanese quails, respectively (Sachdev and Panda, 1989).

In the present experiment, the basal vitamin A level of the feed was 8100 IU/kg. This value was higher than the minimum vitamin A requirement (Ramachandran and Arscott, 1974). This level was supplemented with 50×10^3 IU RA, administered either via the feed (groups A and AC) or via the drinking water (groups AE and AEC). The results indicate that vitamin A content of the egg yolk markedly increased as a result of RA supplementation (Table 2).

In group C receiving ascorbic acid supplementation, the values of the investigated parameters (total number of eggs laid during the experiment, number of eggs per day, mass of whole egg, egg-shell, albumen, and egg yolk, vitamin A concentration of the egg yolk) decreased as compared to the control group. If ascorbic acid was combined with RA (group AC) and both liposoluble vitamins (RA and TA) were given (group AEC), vitamin A concentration of the egg yolk was higher than if only RA (group A) or RA + TA (group AE) was administered.

In view of the numerous health benefits associated with the consumption of antioxidants such as alpha-tocopherol, beta-carotene and retinol, Jiang et al. (1994) conducted an experiment to determine dietary levels necessary for significantly increasing the concentration of these substances in the yolk of chicken eggs. Laying hens were fed on diets containing β-carotene, DL-α-tocopheryl acetate, or their combination at a dose of 0, 50, 100, 200 or 400 mg/kg. Yolk retinol, β-carotene and vitamin E concentrations increased ($P < 0.05$). Yolk retinol concentration increased from 11.6 μg/g in the controls to 13.9 μg/g of yolk in hens receiving β-carotene at 200 mg/kg of diet. Supplemental β-carotene markedly decreased the deposition of α-tocopherol in the yolk when the two compounds were fed in combination. Egg weight and egg yield were not affected by dietary supplementation. Although the data indicated that it was possible to increase the concentration of these fat-soluble vitamins (A and E) and β-carotene in chicken eggs, because of the rather high costs involved the authors concluded that it might not be commercially viable to fortify the egg yolk with β-carotene or retinol by supplementing the diet with β-carotene (Jiang et al., 1994).

The experiment presented in this paper revealed that the different parameters of egg production (number of eggs laid, egg mass, yolk/albumen ratio) were not significantly affected by the supplementations applied (Table 3). At the same time, the combined supplementations (groups AC, AE, AEC) seem to be more effective in terms of vitamin A fortification of the egg yolk. The results indicate that increasing the vitamin A content of the egg yolk by a short-term RA supplementation of the laying ration is a distinct possibility.

The rectification of severe vitamin A deficiencies as well as marginally deficient or suboptimal vitamin A status of children and the elderly is an important public health task in many parts of the world (Underwood, 1984). It is possible that retinoids present in the natural substances of eggs could be a good source of vitamin A for humans. The confirmation of this hypothesis, however, warrants further investigations.

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MOTILITY AND AGGLUTINATION OF FOWL SPERMATOZOA IN MEDIA OF DIFFERENT AMINO ACID CONTENT AND pH VALUE *IN VITRO*

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The objective of the present experiments was to study some properties of fowl spermatozoa which may play a role in the sperm storage and emptying mechanism of the uterovaginal sperm storage tubules (SST) of the hen. The effects exerted by different amino acids (aspartic acid, Asp; glutamic acid, Glu; gamma-aminobutyric acid, GABA; glycine, Gly) and by the pH of the environment at 24 and 39 °C on the motility and agglutination of cock spermatozoa were studied *in vitro*. The spermatozoa did not show agglutination in the presence of Asp and Glu, and became immobilised if the concentration of Glu or the acidity of the environment was increased. In neutral solutions of GABA and Gly or in a faintly alkaline solution characteristic plait-like conglomerations could be seen. The motility of spermatozoa immobilised by Glu could be restored in a varying degree by the addition of GABA or Gly. At a temperature of 24 °C, the spermatozoa became immobilised in a medium of pH 6.0 while showed maximum motility at pH 7.1. At 39 °C, the spermatozoa were immobilised at higher pH (6.2) and required a pH value as high as 7.4–7.5 to show the highest motility. Spermatozoa inactivated in an acidic solution could be immediately mobilised by alkalisation of the medium, irrespective of the Ca^{2+} content of the solution. Thus, Ca^{2+} was not found to play a role in the reactivation of spermatozoa. Nevertheless, marked differences were observed in the maintenance of sperm motility between solutions either containing or lacking Ca^{2+}. As the concentration changes of the above-mentioned amino acids and the pH changes were found to affect the motility and agglutination of spermatozoa *in vitro*, they may influence also the regulatory mechanism of the uterovaginal SST during the egg-formation cycle *in vivo*.

Key words: Fowl, spermatozoa, motility, agglutination, amino acids, calcium, pH, temperature, sperm storage tubules (SST)

The microenvironment of the sperm storage tubules (SST) facilitating the *in vivo* storage of spermatozoa without damage and the effect triggering their release from the SST are not known. Many theories have been proposed as to the cause of storage and release (Zavaleta and Ogasawara, 1987); however, none of them has

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furnished a complete explanation for these phenomena. Their physiological and biochemical bases are yet to be determined. The knowledge of these processes would facilitate the improvement of *in vitro* sperm storage.

The storage and release phenomena are basically related to changes in sperm motility, i.e. the contraction of their contractile elements, which depends on the cell membrane potential. This is primarily determined by the electrolyte concentration of the environment. Therefore, the role played in these processes by transmitter amino acids (aspartic acid, Asp; glutamic acid, Glu; glycine, Gly; gamma-aminobutyric acid, GABA) affecting the transport of Na^+, K^+ and Cl^- in the central nervous system and in other organs (Erdő, 1991) was studied first. Glutamate and aspartate are known to be present in high concentrations in the seminal plasma, in the secretion of accessory sex glands and in the female genital tract of different animal species (Ibrahim and Boldizsár, 1981; Boldizsár et al., 1988). Among the free amino acids present in the oviduct of mammals, amino acids of transmitter character are dominant. In addition, it has been demonstrated that ovulation is accompanied by the secretion of large amounts of taurine (Tau), Gly and GABA in the oviduct (Fahning et al., 1967; Van der Horst and Brand, 1971; Boldizsár et al., 1991). Ashizawa and Okauchi (1984) found that a low molecular weight heat-stable factor of the seminal plasma stimulates sperm motility. The above-mentioned amino acids fulfil these requirements.

As the amino acid solutions represent media of acidic, neutral or slightly alkaline pH, their effect may be explained, besides the transmitter function, also by the pH conditions they create. The motility of fowl spermatozoa is known to be regulated by the intracellular pH (Ashizawa et al., 1994) and the temperature (Munro, 1938; Ashizawa and Sano, 1990). In view of the fact that intracellular pH depends on the pH of the environment, the effect exerted by the pH of media was also examined both at room and at body temperature.

As spermatozoa become deposited with their heads towards the blind end of the tubules in an agglutination-like manner in the SST (Van Krey et al., 1981), the direct effect of the above-mentioned amino acids (experiment 1) and of the pH (experiment 2) on the motility of cock spermatozoa, the occurrence of agglutination forms and the reactivation possibilities of immobilised sperm cells were studied *in vitro*. The results of such studies might furnish an explanation for the immobilisation-mobilisation and agglutination phenomena and sperm reactivation occurring in the sperm storage tubules of the avian oviduct.

The presence of Ca^{2+} in the medium also affects sperm motility (Ashizawa and Wishart, 1987; Wishart and Ashizawa, 1987; Thomson and Wishart, 1988). A further objective of this work was to study the effect of Ca^{2+} on the maintenance of sperm motility and on the reactivation of spermatozoa.

Materials and methods

Semen from 2-year-old New Hampshire cocks kept in individual cages was used for the experiments. The pooled semen samples were diluted tenfold with modified Kiev semen extender (0.1 g NaCl, 2.0 g sucrose, 1.0 g glucose, 0.15 g sodium carbonate, 1.0 g sodium acetate, 0.15 ml 10% acetic acid, distilled water to 100 ml, pH 7.2), centrifuged at 1,500 rpm, then the spermatozoa thus deprived of plasma were again diluted twofold with the same diluent.

Five different solutions were used in the first experiment: (a) Kiev extender as control; diluents containing (b) 2–14 mM Glu; (c) 5–7 mM Gly; (d) 1–8 mM GABA; (e) 1–6 mM Asp. Ten µl of the solution containing amino acids was added to 10 µl of the semen samples in 10 min. The amino acid concentrations of the fowl seminal plasma were taken as a basis when selecting the amino acid concentrations used in this study.

To study the effects of pH in the second experiment, a pH series ranging from 5.4 to 8.6 was prepared from the same extender. The samples were divided into two halves and examined at 24 or 40 °C temperature. To 10 µl aliquots of unwashed, twofold diluted semen samples 10 µl volumes of extender of different pH value were added and the final pHs determined.

Changes in the motility and agglutination of spermatozoa in the presence and absence of the above amino acids and at different pHs were studied by light microscopy for 30 sec, using silicone-coated slides. The tests were always performed by the same person. The intensity of motility was scored from 0 to 5: 0 = all spermatozoa are immotile; 1 = approx. 10% of the spermatozoa are motile, 60 % oscillate, 30% are immotile; 2 = approx. 50% of the spermatozoa oscillate, 50% show slow motion; 3 = approx. 60% of the spermatozoa show a motion of medium intensity, 20 % show progressive motion; 4 = approx. 70% of the spermatozoa show definitely progressive motion; 5 = min. 85% of the spermatozoa show vigorous progressive (vortex) motion. The motility scores were compared by the use of a Hamilton Thorn Research HTM Motility Analyser. After sperm collection and dilution it took about 2 h to finish the motility recording.

In order to demonstrate the effects of Ca^{2+} on the reactivation of spermatozoa, some of the samples were washed with a Ca^{2+}-free extender twice and centrifuged at 1,500 rpm. Prepared from the originally Ca^{2+}-free extender of pH 8.6, a version containing 4 mM Ca^{2+} was also made. The two types of extender were used for reactivating spermatozoa either immobilised or showing poor motility in an acidic medium. Reactivation attempts were made at 24 °C and at 39 °C. Changes in motility were checked as described above.

For the statistical analysis of the data the means, standard deviations, t- or d-test, linear, exponential and polynomial regressions were used (Dixon and Brown, 1979).

Results

In undiluted state the male germ cells showed motility of medium intensity and the appearance of some agglutination clumps of head-to-head configuration in the visual field. In samples diluted 4- to 8-fold with Kiev diluent motility increased while clumping decreased (1-2 agglutination clumps).

In a diluent containing Asp or Glu, the spermatozoa did not show agglutination like those in acidic medium, while in the presence of GABA or Gly they clumped together into characteristic plait-like forms within 30 sec at 24 °C (Fig. 1). In a slightly alkaline medium they formed similar plait-like bundles ranging from loose threads to tight rope-like structures, in which the sperm heads showed an outward deviation from the axis (Fig. 2).

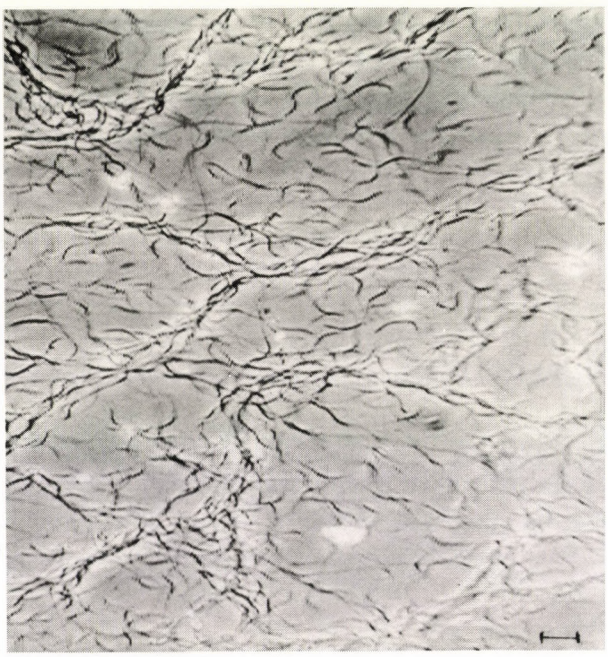


Fig. 1. Characteristic rope-like conglomeration of spermatozoa in the presence of GABA or glycine in the diluent. Bar = 20 μm

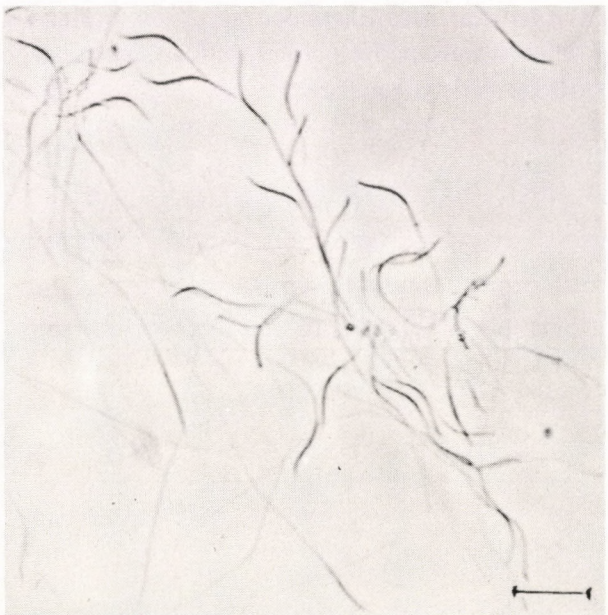


Fig. 2. Loose plait-like agglutination of spermatozoa in a faintly alkaline medium. Bar = 20 μm

In solutions containing Asp the motility of spermatozoa decreases. However, in a 1:1 mixture of Asp and Gly solution a motility scored 4–5 can be observed. Raising the concentration of Glu gradually the motility of spermatozoa decreases and at a concentration of 14 mM Glu the cells become immobilised, but in a 1:1 mixture of Glu and Gly solution a good (score 4) motility occurs, in the same way as in a 1:1 mixture of Glu and GABA solution. The mixture of Asp and Glu arrests sperm motility, while in the presence of Gly the motility of spermatozoa shows a good score. Spermatozoa immobilised with Glu can be reactivated with Gly solution: upon the addition of Gly, 45% of the cells showed expressed progressive motility. If GABA was added to immobilised spermatozoa, 20% showed agile progressive motion while 60% oscillated in one place. The effect exerted on motility was an immediate reaction in both cases (Table 1).

The effect exerted by pH changes on motility as a function of temperature is shown in Fig. 3. The values are situated along a polynomial curve. Motility is arrested at 24 °C under a pH of 5.6–5.8. The most intensive motility can be observed at pH 7.0–7.2, then it decreases and becomes stagnant between pH 7.5 and 8.2. The curve obtained at 39–40 °C is similar to the 24 °C results, but shows a

shift to the right. Spermatozoa are immobilised at pH 6.2 and show maximum motility at pH 7.4. The values above pH 8.0 were not taken into consideration as they are not regarded as physiological ones.

Table 1

Motility of fowl spermatozoa in media of different amino acid content

Amino acids				Means of motility scores
Asp (1.6 mM)	Glu (14 mM)	Gly (5.2 mM)	GABA (4 mM)	
+				3.20 ± 1.16^{a} (n = 4)
+		+		4.60 ± 0.72^{b} (n = 5)
		+		3.80 ± 0.84^{c} (n = 4)
	+			0.50 ± 0.72^{d} (n = 11)
	+	+		4.10 ± 0.54^{e} (n = 5)
	+		+	3.80 ± 0.30^{f} (n = 6)
			+	3.70 ± 0.42^{g} (n = 4)
+	+			0.70 ± 0.60^{h} (n = 3)
+	+	+		3.75 ± 0.32^{j} (n = 3)
Effect of added Gly and GABA solution on the motility of spermatozoa immobilised in Glu solution				
	+	+		3.00 ± 0.88^{k} (n = 3)
	+		+	1.75 ± 0.62^{l} (n = 2)

Note: d–e = $p < 0.01$; d–f = $p < 0.01$; h–j = $p < 0.01$; d–k = $p < 0.01$; d–l = $0.05 < p < 0.1$

Sperm motility arrested in an acidic medium can be also re-initiated by neutralisation of the medium. For reactivation a solution of pH 8.5 was used. This reaction proved to be time-dependent along an exponential curve (Fig. 4). In a basic solution immobilised cells could not be reactivated.

It was surprising to find that while the motility of sperm immobilised with the acidic Glu solution could be restored by adding a neutral Gly solution, a simple alkalisation of the medium failed to produce such an effect, which indicates that transmitter amino acids affect sperm motility not only through their influence exerted on the pH.

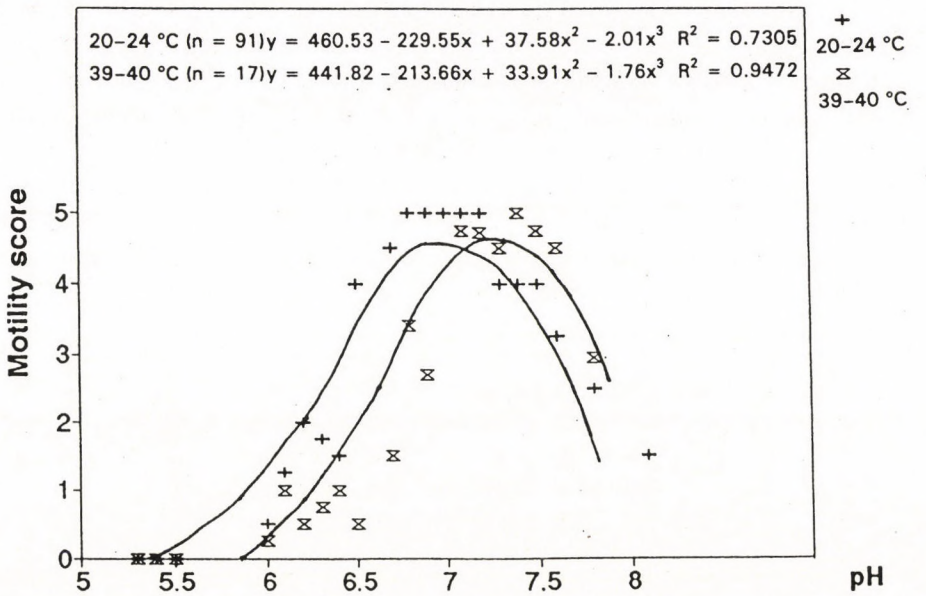


Fig. 3. Relationship between sperm motility and pH at two different temperatures

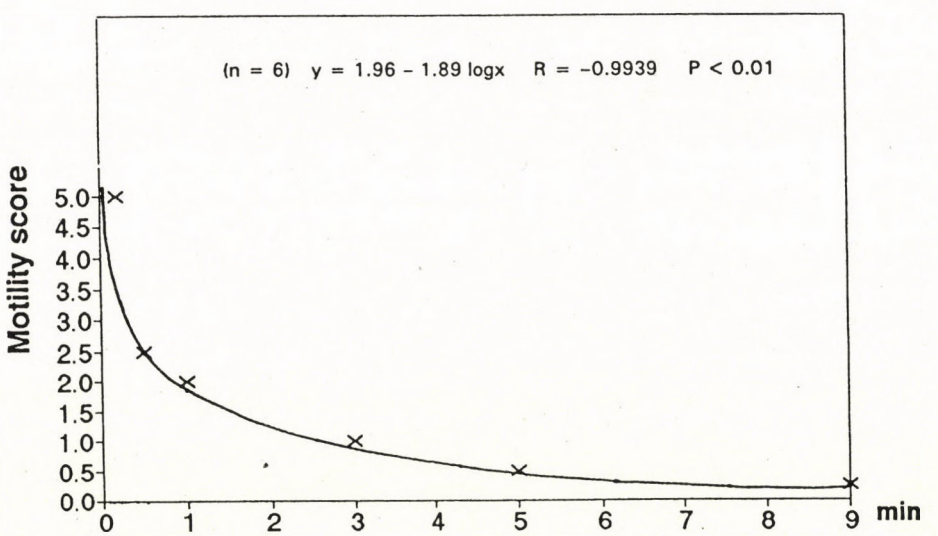


Fig. 4. The possibility of reactivation of immobilised spermatozoa as a function of time (from pH 6.2 by adding an extender of pH 8.5 to the medium)

The same volume of a Ca^{2+}-containing or a Ca^{2+}-free extender of pH 8.6 was added to the sperm showing poor motility in an acidic medium (pH 6.30). At 24 °C a good motility score (5) was obtained, while at 39 °C a somewhat poorer motility (2–3.5) was observed. Ca^{2+} was not found to exert a favourable effect on reactivation (Table 2). In the studies determining how long the reactivated spermatozoa maintain their motility at 24 °C, it was found that in a Ca^{2+}-containing medium they showed satisfactory motility (score 2–3) for about 20 min, while in a Ca^{2+}-free environment their motility markedly decreased after 5 min and ceased after 15 min (Fig. 5).

Table 2

Motility scores of sperm cells at pH 7.1 and 6.67 and after reactivation by adding a solution of pH 8.60 to the medium at 40 ° and 24 °C

Temperature	Unwashed sperm cells	Washed sperm cells			
	pH 7.00–7.10	pH 7.00–7.10	pH 6.67	pH 6.67 + 8.60 (≈ 8.00)	
				Ca^{++}	Ca^{++}-free
40 °C	4.33 ± 0.58 (n = 3)	3.08 ± 1.00[a] (n = 6)	0.75 ± 0.61[b] (n = 6)	2.00 ± 0.00[e] (n = 2)	3.50 ± 1.91[f] (n = 4)
24 °C	4.90 ± 0.22 (n = 10)	4.67 ± 0.58[c] (n = 3)	1.50 ± 1.00[d] (n = 4)	5.00 ± 0.00[g] (n = 2)	5.00 ± 0.00[h] (n = 2)

Note: a–b = $p < 0.01$; c–d = $p < 0.05$; b–e + f = $p < 0.05$; d–g + h = $p < 0.01$; e–f = nonsignificant

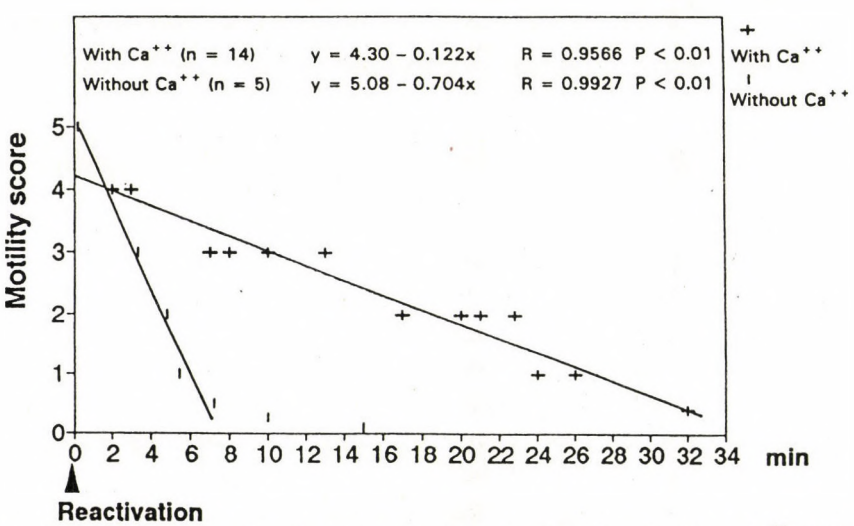


Fig. 5. Changes in the motility of reactivated cells in media with and without Ca^{++}(24 °C)

Discussion

Van Krey et al. (1981) suggest that the mechanism responsible for the storage of spermatozoa in the SST and their evacuation from those tubules is related to agglutination phenomena. Our observations, consistently with the results of the above authors, show that in a fresh ejaculate head-to-head agglutination occurs. Spermatozoa get deposited in the SST parallel to each other (Burke et al., 1972). In the presence of amino acids of acidic character (Glu, Asp) the spermatozoa are evenly and diffusely distributed in the visual field, while in the presence of neutral amino acids (Gly, GABA) they form rope-like conglomerations. The appearance of agglutination forms is probably due to the uneven distribution of, and changes in, the electric potential of the cell membrane in different environments. These factors contribute to the occurrence of agglutination. Through their transmitter functions, the amino acids studied by us may also induce such changes. The pH of the environment has a similar effect on the agglutination of spermatozoa, as it changes the electrolyte concentration of the media and, through that, the membrane potential of the cells.

The reactivation phenomena can be explained by the hyperpolarising effect exerted on the membrane potential by the increased Cl^- transport (Boldizsár et al., 1991). This phenomenon is reminiscent of the "quiescence" induced by CO_2, which can be reversed by perfusion with oxygen (Shettles, 1940; Schindler and Nevo, 1962). The cited authors explain the inactivation of spermatozoa by factors other than a pH change. In the present study inactivation and reactivation of spermatozoa could be achieved also by changing the pH of the medium. It is possible that the observed phenomenon is due to the membrane potential changes induced by different factors in all cases. Harrison and Heald (1966) found poly-α-glutamic acid in a high quantity in the uterovaginal junction and in the infundibulum, which maintained the motility of spermatozoa at body temperature. By decreasing the molecular weight of the poly-α-glutamic acid to 2600, however, the motility also decreased and ceased. Sliwa (1990) found that glutamic acid, if present in a low concentration, enhanced the motility of mouse spermatozoa; at the same time, when applied at a higher concentration, it reduced the motility of cells in the same way as did alanine, glycine, arginine and leucine.

Thomson and Wishart (1988) compared the motility of spermatozoa at temperatures of 30 °C and 40 °C. The sperm cells were found to be motile at 30 °C and immotile at 40 °C. Existing data of the literature are inconsistent as to the effect exerted by pH on motility (Ishikawa, 1930; Bogdanoff and Shaffner, 1954). At room temperature, Wilcox and Shaffner (1957) observed the highest motility at a pH of 6.75, which is in contrast with the results of our study. That

difference can be explained by the room-temperature differences in the two experiments.

We found that fowl spermatozoa show the most intensive motility at 24 °C. Therefore, we used this temperature and the body temperature (39–40 °C) for studying the motility of spermatozoa in solutions of different pH value.

A pH variation of 0.3 (7.2–7.5) has been reported to occur on the uterine mucosa in the oviduct of the hen during the egg formation cycle (Winget et al., 1965). Such variation may be even more expressed in the interstitium and lumen of the SST, especially if we consider the special, dual blood supply system of these tubules (Gilbert et al., 1968). As some of the capillaries supplying the SST are derived from the uterine veins, the pH of the blood plasma is more acidic in them and their Ca^{++} content is lower at the time of egg shell formation than those of the blood plasma in other capillaries found in the oviduct. We detected large quantities of Glu, Tau and Asp in the washing fluid of the oviduct, and observed that their quantity abruptly dropped after oviposition (Barna et al., 1996). This is the time when spermatozoa leave the sperm storage tubules in order to fertilise the ovum.

In studies on fish spermatozoa, Morisawa et al. (1983) also found that acidic media inhibit sperm motility while alkalisation activates it. Gatti et al. (1990) reported that the plasma membrane potential of rainbow trout spermatozoa is sensitive to the hydrogen ion transmembrane gradient and that intracellular pH exerts an influence on sperm motility. The same relationship had been proved by numerous researchers in invertebrates as well.

Thomson and Wishart (1988) explained the difference found in sperm motility at 30 °C and 40 °C by the loss and uptake of Ca^{2+}. By adding seminal plasma to washed sperm immobilised at 40 °C, Nevo and Schindler (1968) could reactivate the spermatozoa, which they also attributed to an effect of Ca^{2+} where the amino acid composition and the slightly alkaline pH of the seminal plasma may also have played a role. In the present study, Ca^{2+} was not found to play a role in reactivation; however, at a temperature of 24 °C sperm motility could be maintained for a longer time in the presence of Ca^{2+}. It can be assumed that intracellular free Ca^{2+} may account for the dissimilar behaviour of spermatozoa at the two temperatures tested.

Besides several other factors such as immune proteins, the existence of different agglutination forms of spermatozoa in media of the tested pHs, amino acid contents and dilutions can be explained by changes of the membrane potential at different parts of the sperm cell. The special agglutination arrangement of spermatozoa at a mildly acidic pH in the SST can contribute to the regulation mechanism of storage and release. During egg shell formation, an accumulation of citrates, acid phosphatase and mucopolysaccharidase can be observed in the wall of

the uterus. Due to these facts and to the action of the carbonic anhydrase enzyme the pH of the venous blood shifts towards the acidic range (Mongin, 1968). All these events can be assumed to cause an acidic shift in the pH of the environment surrounding the spermatozoa stored in the SST, a change which — according to our studies — results in the immobilisation and impaction of sperm cells. Once egg shell formation is completed, this effect ceases.

Acknowledgement

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AMINO ACID SECRETION OF THE HEN'S OVIDUCT DURING THE EGG FORMATION CYCLE

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The free amino acid content of 34 hens' oviducts was determined in different phases of the egg formation cycle. In addition, quantitative changes of transmitter-type amino acids (glutamic acid, aspartic acid, taurine, glycine) were monitored in comparison with those of other amino acids participating in protein synthesis (e.g., lysine, tyrosine, valine). Different portions of the hen's oviduct, primarily the magnum and the uterus, were characterised by substantial amino acid secreting activity. The amino acids detectable in the highest quantity were taurine, glutamic acid, glycine and aspartic acid. These amino acids were present in high concentrations throughout the egg formation cycle, but their quantity abruptly decreased at the time of oviposition and ovulation. The quantitative changes of transmitter-type amino acids are mostly independent of those of other amino acids participating in protein synthesis. Since in our earlier *in vitro* studies the concentration changes of the transmitter amino acids studied were found to affect the motility and agglutination of spermatozoa, they — besides other factors — can be assumed to exert an influence on the sperm storage and release processes of the uterovaginal sperm storage tubules located in the oviduct of birds.

Key words: Hen, oviduct, amino acid, sperm storage tubules (SST)

The objective of this study was to determine the amino acid composition of the secretion of the hen's oviduct, followed by the demonstration of concentration changes of the amino acids during the egg formation cycle and the quantitative differences between the transmitter-type amino acids (glutamic acid, Glu; aspartic acid, Asp; glycine, Gly; taurine, Tau; gamma-aminobutyric acid, GABA) and other amino acids participating in protein synthesis.

In earlier studies we examined the effect of transmitter-type amino acids on the motility and agglutination forms of spermatozoa *in vitro*. In a diluent supplemented with glutamic acid and aspartic acid, i.e. amino acids of acidic character, spermatozoa became immotile and did not show agglutination in the visual field. If neutral amino acids (Gly, GABA) were added to the medium within a short time, the cells regained their motility. In the presence of high concentrations of the latter

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amino acids the spermatozoa became arranged in plait-like bundles (Barna and Boldizsár, 1996).

Amino acids of transmitter character occur in strikingly high concentrations in the seminal plasma of mammals (Ibrahim and Boldizsár, 1981; Boldizsár et al., 1988) and birds (especially glutamic acid; Lake, 1971). Their presence in the uterine secretion and uterine wall of mammals has also been demonstrated (Fahning et al., 1967; Erdő et al., 1982; Erdő, 1984).

The aim of these studies was to support the hypothesis that the concentration changes of these amino acids supposedly affect the motility of spermatozoa in the oviduct as well as their deposition into, and release from, the sperm storage tubules (SST).

Materials and methods

A total of 34 individuals of 1.5-year-old New Hampshire laying hens kept in separate cages were used for the experiments.

To determine the free amino acid content of entire oviducts at various stages of egg formation, 14 hens were killed 0–0.5, 2–5, 7–8, 14–16 or 20–22 h after oviposition. The oviducts, being either empty or containing eggs in various portions, were removed and flushed with 10 ml of distilled water. The washing fluid was deproteinised by the addition of 10% sulphosalicylic acid, then centrifuged at 2,500 rpm for 10 min. The amino acid content of the supernatant was determined in a Biocal single-column amino acid analyser.

Different portions of the oviduct (infundibulum, magnum, isthmus, uterus, vagina) of 7 other hens at different stages of the egg formation cycle were rinsed with 2 ml distilled water each, then processed for the determination of free amino acid content as described above.

The amino acid secretions of the oviducts in different functional phases were also studied in a similar manner on 13 laying hens: (1) inactive, (2) before the onset of oviposition, (3) during active oviposition, (4) at the end of oviposition.

Data were statistically analysed by One-Sample Analysis.

Results

The average content of free amino acids found in the washing fluid of the *entire oviduct* during the egg formation cycle is shown in Fig. 1. A substantial amino acid secretion was demonstrable in the hen's oviduct at various stages of the egg formation cycle. Taurine and glutamic acid were present in outstandingly high concentrations but glycine and aspartic acid also occurred in concentrations higher than those of the other amino acids.

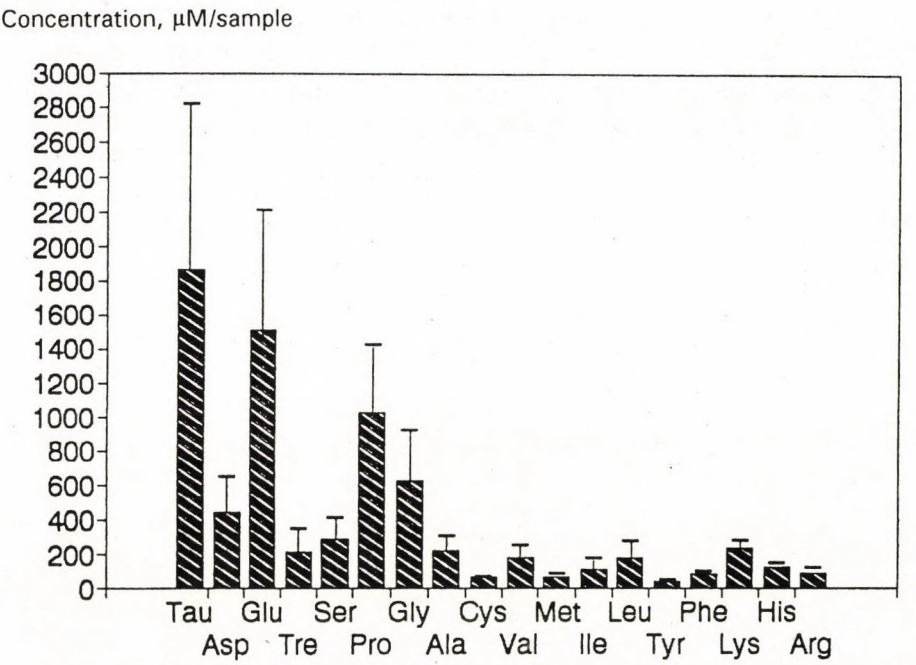
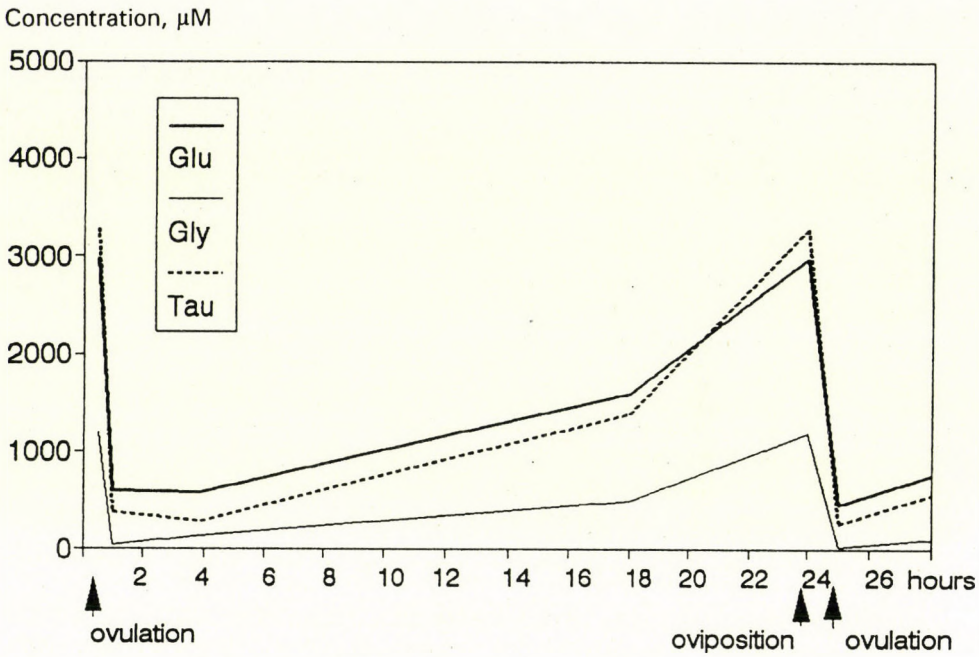


Fig. 1. Mean free amino acid content of the washing fluid of the hen's oviduct during the egg formation cycle (n = 8)

Figure 2 shows quantitative changes of the three amino acids present in the highest concentration during the 25-hour egg formation cycle. The concentration of all three amino acids rose from the 1st to the 24th hour but abruptly decreased at the time of oviposition and ovulation. This is the time when spermatozoa leave the sperm storage tubules in order to fertilise the ovum.

Glutamic acid, taurine and glycine were the dominant amino acids in the secretion of different *portions* of the oviduct as well (e.g., in the uterus: Glu = 0.526 ± 0.362, Tau = 0.468 ± 0.364, Gly = 0.161 ± 0.112, and in the magnum: Glu = 0.443 ± 0.332, Tau = 0.487 ± 0.473, Gly = 0.077 ± 0.076). Lysine was also found in relatively high concentrations (Table 1). The highest values were obtained for the magnum and uterus, while minimal amounts occurred in the vagina, isthmus and in some cases in the infundibulum. The amino acid concentrations found in the different portions of the oviduct are in good agreement with the values obtained for the oviduct as a whole. These reached the highest concentration before ovulation (empty oviduct), primarily in the uterus and magnum.



Tau =	367 ± 222.03	288	1386 ± 330.22	3269 ± 1257.24
Glu =	590 ± 494.97	570	1600 ± 424.36	2969.5 ± 1619.98
Gly =	74	138	495 ± 309.31	1187.5 ± 823.77

Fig. 2. Quantitative changes in the concentration of transmitter amino acids during the egg formation cycle

The amino acid secretion of oviducts in different functional phases (inactive phase, different phases of active oviposition, end of oviposition) was also compared (Table 2). The secretory activity was found to be increased in the oviduct commencing its phase of activity. It reached its peak at the time of active oviposition, and dropped to the inactive level immediately after the end of oviposition.

As intensive protein synthesis is conducted in the oviduct of birds over a period of several hours during egg formation, the concentration changes of amino acids regarded as indicators of protein synthesis (lysine, valine, tyrosine) were compared with those of glutamic acid, a transmitter-type amino acid. Figure 3 shows the glutamic acid/lysine and glutamic acid/valine ratios in different oviduct portions during the egg formation cycle. As compared to both lysine and valine, glutamic acid is present in high concentrations in all portions of the oviduct, but especially in the magnum and uterus.

Table 1

Mean amino acid concentrations of different portions of the oviduct at different stages of the egg formation cycle (μM/sample)

Amino acids	Oviduct portion														
	Infundibulum			Magnum			Isthmus			Uterus			Vagina		
	a*	b	c	a	b	c	a	b	c	a	b	c	a	b	c
Tau	**0.340**	0.096		**1.000**	0.066	**0.396**	**0.230**	0.030	0.086	**0.750**	0.057	**0.599**	0.060	0.039	0.024
Glu	**0.120**	**0.220**	0.083	**0.550**	0.071	**0.710**	**0.230**	0.055	0.057	**0.860**	0.140	**0.580**	0.064	0.034	0.035
Asp	0.029	0.025	0.038	0.092	0.014	0.087	0.055	0.024	0.038	0.260	0.067	0.170	0.049	0.010	0.014
Gly	**0.054**	0.025	**0.047**	**0.160**	0.010	**0.062**	**0.086**	0.015	**0.025**	**0.280**	0.055	**0.150**	0.024	0.032	0.023
Lys	0.460	0.156	0.360	0.370	0.024	0.330	0.370	0.300	0.048	0.065	0.340	0.450	0.300	–	0.350
Tre	traces	traces	0.024	traces	traces	0.035	traces	traces	traces	0.018	0.021	0.039	traces	traces	traces
Ser	0.014	0.054	0.091	0.015	0.001	0.200	0.017	0.031	0.036	0.037	0.080	0.002	0.014	0.043	0.026
Ala	traces	traces	0.014	–	traces	0.027	–	0.005	traces	–	0.010	0.019	–	0.005	–
Cys	–	–	–	–	–	0.070	–	–	–	–	–	–	–	–	–
Val	0.056	0.036	0.058	–	0.018	0.056	0.017	0.050	0.049	–0.087	0.025	0.064	0.015	0.018	0.037
Met	–	–	–	–	–	0.038	–	–	–	–	–	–	–	–	–
Ile	–	–	–	–	–	0.003	–	–	–	–	–	0.021	–	–	–
Leu	–	–	–	–	–	0.008	–	–	traces	–	0.043	traces	traces	–	traces
Tyr	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Phe	–	–	–	–	–	–	–	–	–	–	–	0.034	–	–	–
His	–	–	–	–	–	–	–	–	–	–	0.099	–	–	–	–

*Position of egg: a = before ovulation; b = egg in the first third of magnum; c = egg with calcic shell in the uterus

Table 2

Amino acid secretion of the oviduct at different stages of the oviposition cycle (μM/sample) (n=12)

Amino acid	Oviduct stage				
	Inactive oviduct	Immature follicle on ovary	Mature follicle on ovary	Active oviposition	End of oviposition
Tau	0.145 ± 0.021	0.550	0.710	1.863 ± 0.960	0.210
Glu	0.165 ± 0.000	0.130	1.240	1.511 ± 0.708	0.245
Asp	0.090 ± 0.014	0.030	0.500	0.452 ± 0.204	0.109
Gly	0.055 ± 0.000	0.100	0.380	0.629 ± 0.301	0.074
Tre	0.070 ± 0.056	0.060	0.230	0.210 ± 0.148	0.156
Ser	0.135 ± 0.049	0.190	0.070	0.292 ± 0.125	0.071
Ala	0.025 ± 0.000	–	0.130	0.223 ± 0.090	0.166
Val	0.035 ± 0.000	–	0.140	0.181 ± 0.079	0.217
Met	traces	–	0.057	0.065 ± 0.025	0.030
Ile	traces	–	0.091	0.113 ± 0.067	0.035
Leu	0.027 ± 0.000	–	0.113	0.180 ± 0.107	0.020
Lys	0.033 ± 0.000	–	–	0.239 ± 0.053	0.056

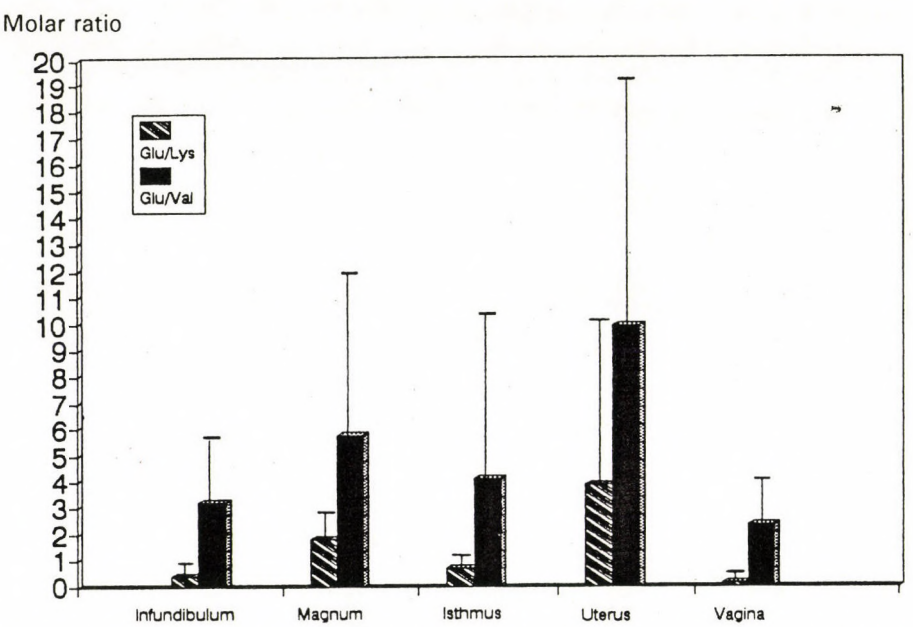


Fig. 3. Mean Glu/Lys and Glu/Val concentration ratios in the different oviduct portions during the entire cycle (n = 4)

Discussion

The secretion of most amino acids present in the oviduct may be a concomitant of intensive protein synthesis associated with egg-white formation. Some amino acids also have transmitter functions through which they can exert an influence on sperm motility and on the mechanism of sperm release from the SST.

Glutamic acid and aspartic acid, and the similarly highly acidic taurine and hypotaurine, were found to occur in the oviductal secretion in high concentrations irrespective of protein synthesis, i.e., they may have also other functions. Considering the fact that in the female genital tract the male germ cell loses the seminal plasma, the high glutamic acid content of the oviduct is surprising. In view of the presumably small volume of the oviductal secretion, this value exceeds the extremely high glutamic acid concentration that can be measured in the seminal plasma. The high concentration of Glu measured in the magnum during protein synthesis can easily be accounted for by the high Glu content of the egg-white (approximately 26% of the egg-white is constituted by acidic amino acids, i.e., Asp and Glu; FAO, 1970), but the similarly high Glu concentration obtained in the uterus cannot be explained on that basis.

Glycine, a neutral amino acid, was also found in substantial quantities. Taurine is an amino acid metabolite that does not participate in building proteins: its transmitter role has been suggested (Van der Horst and Brand, 1971; Kuriyama, 1980). It is present in strikingly high concentrations both in the seminal plasma of different species and in the secretion of the oviduct. The role played by taurine in these biological secretions is not known yet. As it is assumed to act as an intracellular Ca^{2+} mobiliser and is known to be involved in capacitation, acrosomal reaction and penetration, its direct effect exerted on male germ cells is conceivable.

According to numerous authors, the concentration changes of the amino acids studied by us are under hormonal regulation in different species. The amino acid content of the seminal plasma is controlled by testosterone (Horváth and Solymos, 1954; Krampitz and Doepfmer, 1962; Kochakian, 1973; Hernvann et al., 1986).

Glycine secretion in the female genital tract has also been demonstrated to be under hormonal regulation in rabbits (Gregoire et al., 1961) and cattle (Fahning et al., 1967). Van der Horst and Brand (1971) established that the secretion of taurine and hypotaurine is regulated by progesterone. Oestrogens have been reported to increase the transport of alanine, glutamic acid, lysine, proline and serine (Manchester, 1976).

In the laying hen, the cyclic changes of progesterone, 17β-oestradiol and testosterone hormone levels accompanying egg formation are followed — with a

few hours' delay — by changes in the secretion of transmitter amino acids. The level of steroid hormones reaches the peak before oviposition; after ovulation it markedly decreases, then starts to gradually rise again (Péczely, 1987). This indicates that amino acid secretion is closely correlated with sexual activity.

In the magnum, most amino acids are present in high concentrations because of the protein synthesis taking place there. It is a conspicuous finding that in the uterus only the transmitter amino acids and lysine occur in high concentrations. With a view to the unique dual blood supply of the SST (Gilbert et al., 1968*b*), it may be supposed that the acidic environmental influence (glutamic acid, aspartic acid, citrates, acid phosphatase, carbon dioxide) accompanying the egg formation process makes its effect felt also in the microenvironment of the SST. This effect results in a temporary arrest of sperm motility, in the same way as *in vitro* perfusion with CO_2 is known to do. As phenylalanine and tyrosine, the typical indicators of protein synthesis, could not be measured in some portions, the concentrations of lysine and valine were taken as a basis in the comparative evaluation of glutamic acid fluctuations (Fig. 3).

We could not detect GABA in the secretion of the oviduct, although Erdő (1984) found it in very high concentrations in the wall of the Fallopian tube of mammals and showed it to be under hormonal regulation. GABA type binding sites were demonstrated on the membranes of spermatozoa (Erdő and Wekerle, 1990). The latter was confirmed also by ourselves in a study of Cl^- transport in the presence of GABA (Boldizsár et al., 1991).

The marked decline in the concentration of acidic amino acids at the time of ovulation coincides with the stoppage of calcic egg-shell formation and with the reduction of carbon dioxide content accompanying that high activity conducted in the tissues. At the same time, in the SST neutral lipids are secreted (Gilbert et al., 1968*a*); all these changes shift the ambient pH of the SST towards the neutral range which, according to our earlier studies (Barna and Boldizsár, 1996), is optimal for sperm motility. This is also likely to assist in the release of sperm from the sperm storage tubules.

In our view, the decisive component of sperm release follows the cyclicity of oviposition and ovulation in laying birds. During a one- or two-day break in egg-laying the efflux of spermatozoa may remain continuous, as the acidic metabolites accompanying calcic egg-shell formation and protein synthesis occur in minimal concentrations.

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DETECTION OF HOMOLOGOUS DNA SEQUENCES IN ANIMAL ADENOVIRUSES BY POLYMERASE CHAIN REACTION

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A primer pair originally designed for the universal detection of human adenovirus (HAV) serotypes of all subgenera was modified then tested and found feasible for the detection of different bovine, ovine, and porcine adenovirus (BAV, OAV, and PAV, respectively) serotypes. Apparently, in the examined viruses, parts of the DNA sequence coding for the basal part of the hexon protein are conserved enough for being applicable in polymerase chain reaction (PCR) as primers. Positive amplification could be obtained even from the so-called subgroup 2 BAVs, which viruses do not cross react with HAVs or subgroup 1 BAVs in Southern hybridisation.

Key words: PCR, primer, bovine, ovine, porcine, adenovirus, hexon gene

The DNA restriction enzyme (RE) analysis of different serotypes of bovine (Kurokawa et al., 1978; Belák et al., 1983; Hu et al., 1984*b*; Benkő et al., 1988; Benkő and Harrach, 1990), porcine (Garwes and Xuan, 1989; Benkő, 1990; Kleiboeker et al., 1993; Reddy et al., 1993; Reddy et al., 1995; Tuboly et al., 1995), and ovine (Benkő, 1990; Boyle et al., 1994) adenoviruses (BAVs, PAVs, and OAVs, respectively) did not indicate the existence of very close genetic relationships among these animal adenoviruses and any types of human adenoviruses (HAVs). The only exception was BAV-9, with RE patterns resembling those of group C HAVs (Benkő, 1990). Southern hybridisation experiments revealed DNA homology between HAVs, BAVs, and PAVs, except subgroup 2 (a serologically and biologically different cluster) BAVs (Bartha, 1969), which showed no homology to HAVs or subgroup 1 BAVs (Benkő, 1990; Benkő et al., 1990). Sequence data of bovine adenoviruses available to date suggest that the genome organisation, the relative size and location of some important genes, especially those of the

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genes of the late mRNAs, are comparable in the human and animal adenoviruses (Hu et al., 1984*a*; Mittal et al., 1992; Elgadi et al., 1993; Esford and Haj-Ahmad, 1994; Salmon and Haj-Ahmad, 1994).

A primer pair and a PCR method were earlier elaborated for the general detection of HAVs (Allard et al., 1990). The 301 base pair (bp) long sequence flanked by the two primers (HexAA1885 and HexAA1913) encodes for the basal part of the β-barrel-forming P1 domain of the hexon protein (Roberts et al., 1986). The DNA sequence of the primers was taken from the most conserved parts of this region, where all the known HAV hexon sequences were identical. By the use of these primers, all the 18 examined HAV serotypes representing all the six subgenera A through F could be detected by PCR (Allard et al., 1990). Based on the available sequence data of bovine adenoviruses, we have altered these general human adenovirus primers by introducing some degeneracy.

In the present work, the modified primers were tested successfully on certain adenoviruses of animal origin.

Materials and methods

Source of viruses. The designation of virus strains examined are listed in Table 1. The references of prototype BAVs, PAVs, and OAVs were recently reviewed by Benkő (1990), Derbyshire (1989), and Belák (1990), respectively. Besides the prototype strain of BAV-10, four additional isolates from Northern Ireland (Adair et al., 1996) were included in our studies.

Preparations of viral DNA. Phenol-extracted and alcohol-precipitated viral DNA (at a concentration of 6 to 10 μg/ml estimated on agarose gels), as well as crude preparations of adenovirus infected cell lysates were used. The DNA extraction methods were described earlier (Benkő et al., 1988). Crude preparations were made from inoculated test tube cultures. When the cytopathic effect was maximal, the tubes were frozen and thawed twice, then 0.5 ml of each cell suspension was boiled in an Eppendorf tube for 10 min. After 5 min of centrifugation at maximum rpm in a microcentrifuge, 1 μl of the supernatants were used in the PCR assay. For control purposes, uninfected MDBK cell cultures were treated in the same way. The viral DNA of PAV-5 was kindly provided by T. Tuboly.

Primers. The nucleotide sequence of the 25-mer left primer (HexAA1885) and the 23-mer right primer (HexAA1913) designed for HAV detection is shown in Table 2. Taking into consideration the known hexon gene sequence of BAV-3 (Hu et al., 1984*a*) and the earlier observed AT-rich coding strategy of BAV-4 (Benkő and Harrach, 1994) we have altered these primers by introducing wobbles at positions 7 and 13 (left primer: HexAdB), and at positions 5, 6, 7, and 22 (right

primer: HexAdJ), as shown in detail in Table 2. Thus the extent of degeneracy was four- and sixteen-fold in the left and right primers, respectively. In the degenerate primers, however, the sequence of coded amino acids was preserved, except the 5' end of the right primer, where even BAV-3 and the HAVs contain different amino acids (Toogood and Hay, 1988). The primers were synthesized on a Gene Assembler Special oligonucleotide synthesizer (Pharmacia LKB), and their concentration was measured by a GeneQuant RNA/DNA Calculator (Pharmacia LKB).

Table 1

Designations and recent references of the adenovirus strains examined

Serotype	Subgroup	Isolate	Reference	
BAV-1	1	B-10	Benkő et al.	1988
BAV-2	1	B-19	Benkő et al.	1988
BAV-3	1	WBR-1	Benkő et al.	1988
BAV-4	2	THT/62	Benkő et al.	1988
BAV-5	2	B4/65	Benkő et al.	1988
BAV-6	2	671130	Benkő et al.	1988
BAV-7	2	Fukuroi	Benkő et al.	1988
BAV-8	2	285	Benkő et al.	1988
BAV-9	1	Sofia-4/67	Benkő et al.	1988
BAV-10	2?	Ruakura	Horner et al.	1989
BAV-10	?	Belfast 1	Adair et al.	1996
BAV-10	?	Belfast 2	Adair et al.	1996
BAV-10	?	Belfast 3	Adair et al.	1996
BAV-10	?	Belfast 4	Adair et al.	1996
OAV-1		S1	Belák	1990
OAV-2		PX515	Belák	1990
OAV-3		PX611	Belák	1990
OAV-4		7769	Belák	1990
OAV-5		SAV	Belák	1990
OAV-6		WV419	Belák	1990
PAV-1		25R	Derbyshire	1989
PAV-3		6618	Derbyshire	1989
PAV-5		HNF70	Hirahara et al.	1990

Polymerase chain reaction. The PCR was carried out in 50 µl of reaction mixture containing 5 µl of 10 × buffer (500 mM KCl, 15 mM $MgCl_2$, and 100 mM Tris-HCl, pH 9.0), 50 pmol of each primer, 1 U of Taq DNA polymerase,

300 μM of each of the four deoxynucleoside triphosphates (dATP, dCTP, dGTP, and dTTP; Pharmacia LKB), and 1 to 5 μl of the target DNA. The volume of the reaction mixture was adjusted to 50 μl by adding distilled water. For positive control, HAV-2 total genomic DNA was used, while a lysate of uninfected MDBK cells served as negative control.

Table 2

DNA sequence of the PCR primers

Primer	Sequence	Amplimer length (bp)
HexAA1885	5' GCCGCAGTGGTCTTACATGCACATC 3'	301
HexAdB	5' GCCGCAGTGGTCTTACATGCACATC 3' A C	301
HexAA1913	5' CAGCACGCCGCGGATGTCAAAGT 3'	301
HexAdJ	5' CAGCACGCCGCGGATGTCAAAGT 3' GTA A	301

HexAA1885 and HexAA1913 are the original primers designed for the detection of human adenoviruses, while HexAdB and HexAdJ are the modified (left and right) primers used in this work. Underlined letters show the nucleotides at which wobbles were allowed. The alternative bases are presented below the original sequence.

DNA amplification was repeatedly carried out in two machines: either in a DNA Thermal Cycler (Perkin Elmer Cetus) at the Veterinary Institute of Debrecen, or in a PDR 91 DNA reproducer (BLS Ltd., Hungary) at the Veterinary Medical Research Institute, Hungarian Academy of Sciences, in Budapest. The first cycle consisted of denaturation of the target DNA at 94 °C for 10 min, annealing at 55 °C for 30 sec, and polymerisation at 72 °C for 30 sec. Cycles 2 to 34 consisted of the same three steps, but only 30 sec denaturation time was applied. For the last, 35th cycle, an extended elongation step (72 °C) of 5 min was used. In several experiments, lowered annealing temperatures (45 and 50 °C) were also tested.

Detection of PCR products. The amount and size of the amplification products were analysed by electrophoresis of 8 μl of the reaction mixture on a 1.5% agarose gel (SeaKem) in TBE buffer containing ethidium bromide

(0.5 μg/ml), at a constant voltage of 110 V for 45 min. For molecular weight marker *Pst*I cleaved λ phage DNA (Fermentas, Lithuania) was loaded on the same gel. The gel was visualised by ultraviolet light at 302 nm wavelength and photographed on Polaroid 665 and/or 667 films.

Results

Agarose gels after electrophoresis of the products of different PCRs performed with an annealing temperature of 55 °C are shown in Figs 1 and 2. As can be seen, specific amplification of an approximately 300 bp DNA fragment could be achieved using the modified primers from each of the examined animal adenovirus serotypes. A variation in the intensity of the bands can, however, be observed, indicating that the amount (final concentration) of the amplification products is not equal in each reaction. Less product was consistently obtained when testing the members of the subgroup 2 BAVs (Fig. 1). Also, the performance of crude preparations was generally inferior to that of purified DNA (Fig. 2, lanes 2 and 3).

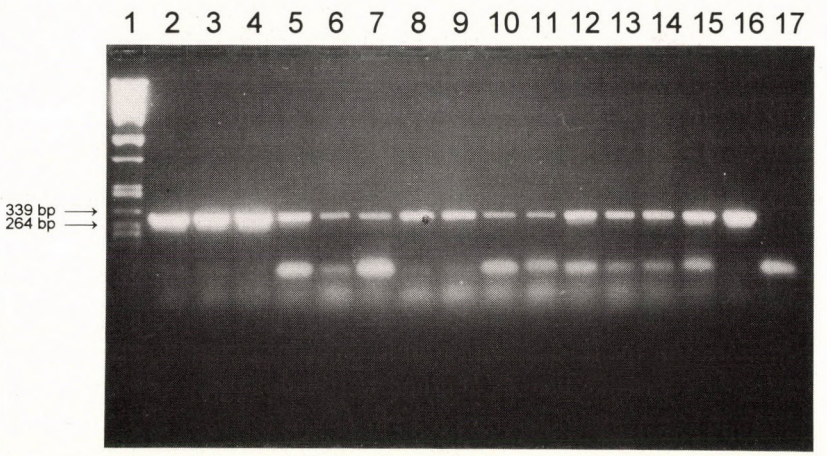


Fig. 1. Gel electrophoresis of the PCR amplification products from different bovine adenovirus serotypes with primers HexAdB and HexAdJ. Lane 1: Molecular weight marker: λ phage DNA digested with *Pst*I, fragment sizes as marked; Lane 2: BAV-1 DNA; Lane 3: BAV-2 DNA; Lane 4: BAV-3 DNA; Lane 5: BAV-9 infected cell lysate; Lane 6: BAV-4 infected cell lysate; Lane 7: BAV-5 infected cell lysate; Lane 8: BAV-6 DNA; Lane 9: BAV-7 DNA; Lane 10: BAV-8 DNA; Lane 11: prototype BAV-10 (strain Ruakura) infected cell lysate; 12-15: DNA of the different isolates of BAV-10, namely: Belfast-3, Belfast-2, Belfast-4, and Belfast-1, respectively; Lane 16: Positive control: HAV-5 DNA; Lane 17: Negative control: uninfected MDBK cell lysate

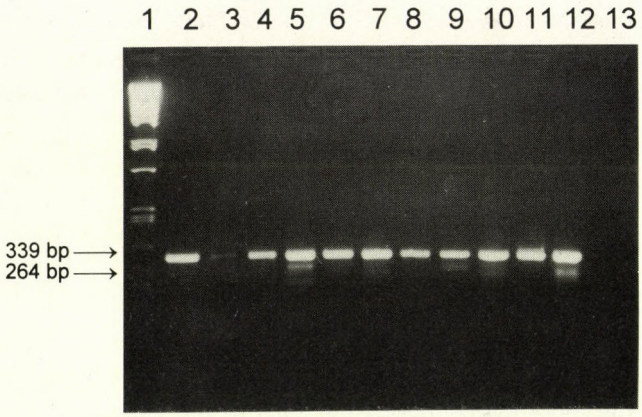


Fig. 2. Gel electrophoresis of PCR amplification products from ovine and porcine adenoviruses. Lane 1: Molecular weight marker: λ phage DNA digested with *PstI*; Lane 2: OAV-6 DNA; Lane 3: OAV-6 infected cell lysate; Lane 4: OAV-5 infected cell lysate; Lane 5: OAV-1 DNA; Lane 6: OAV-2 DNA; Lane 7: OAV-3 infected cell lysate; Lane 8: OAV-4 infected cell lysate; Lane 9: PAV-5 infected cell lysate; Lane 10: PAV-1 DNA; Lane 11: PAV-3 DNA, Lane 12: Positive control: HAV-5 DNA; Lane 13: Negative control: uninfected MDBK cell lysate

Discussion

Recently, up-to-date molecular biological techniques such as DNA hybridisation, restriction fragment length polymorphism (RFLP) analysis, or *in vitro* amplification of DNA by PCR are being applied in identifying microorganisms of interest. In the present study, we have used BAVs, OAVs, and PAVs as models to test the applicability and feasibility of PCR in the detection of different animal adenovirus serotypes. In preliminary experiments (Benkő, 1990; Allard, 1992), a primer pair designed for the general detection of HAVs was found feasible for the detection of bovine, ovine, and porcine adenoviruses. The identity of the amplification products was then confirmed even by Southern-blot hybridisations (Benkő, 1990). However, a significant reduction was observed in the yield of the amplification products resulting from the subgroup 2 BAV serotypes. This reduced yield could partially be overcome by reducing the annealing temperature to 45 °C (Benkő, 1990).

In order to enhance the performance of the human primers, some alterations have been introduced into the primers on the basis of the presently known animal adenovirus DNA sequences (Hu et al., 1984*a*; Benkő and Harrach, 1994). With the modified primer pair, amplification products similar in size and quantity could be obtained from HAV-2 and from the examined serotypes of porcine, ovine, and

subgroup 1 bovine adenoviruses. The yield of PCR products resulting from subgroup 2 BAVs was still slightly less (Fig. 1), but superior to the amount obtained earlier with the original human primers (Benkő, 1990).

Our experiments produced further evidence of the existence of well-preserved coding regions in the hexon gene of different animal adenoviruses. This finding might be exploited in orientating restriction site maps of adenovirus genomes, since selected clones (or separated DNA fragments) can directly be tested by PCR for the presence of the hexon-coding gene. Moreover, this PCR procedure might also be introduced into phylogenetic studies. Computer-aided phylogenetic analysis can be performed on nucleic acid and protein sequence data of limited size if they originate from corresponding genomic regions (Bailey and Mautner, 1994). For this purpose, however, further modification of the method, i.e. selection of other primers flanking longer stretches of DNA, might be needed.

An obvious field of practical application of the described PCR method would be, in veterinary diagnostics, the detection of adenoviruses which are difficult to isolate and grow. These viruses comprehend above all the members of subgroup 2 BAVs, several OAVs, and especially the most recently described BAV-10, which virus seems to bear outstanding pathological importance (Benkő et al., 1995; Smyth et al., 1996). The applicability and performance of the method on clinical samples needs to be evaluated, and a possibly simple procedure for the pre-treatment of clinical samples appropriate for routine laboratory use has to be elaborated.

In veterinary diagnostics, type classification of the detected adenoviruses might be of interest. According to the results of several pilot experiments on BAVs, restriction endonuclease digestion and subsequent gel electrophoresis of the cleaved amplification products (Allard, 1992) might be feasible in type identification. So far, we have tested REs with short recognition sequences (frequent cutters, such as *Alu*I, *Msp*I, *Rsa*I), and obtained promising results. Nevertheless, for such purposes, another PCR method resulting in larger (500–1000 bp long) products might also be indispensable.

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INFECTIOUS CANINE HEPATITIS: DETECTION OF CANINE ADENOVIRUS TYPE 1 BY POLYMERASE CHAIN REACTION

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A primer pair and a polymerase chain reaction (PCR) method earlier tested for the detection of human and animal adenoviruses were used to demonstrate the presence of canine adenovirus type 1 (CAV-1) in tissue culture and clinical specimens. A simple procedure of sample preparation was elaborated making the PCR easily applicable in rapid confirmation of the diagnosis of infectious canine hepatitis.

Key words: Infectious hepatitis, PCR, canine adenovirus

Canine adenovirus type 1, the causative agent of infectious canine hepatitis, can provoke severe disease in dogs and other carnivores. Death from infectious canine hepatitis is usually sporadic, although small outbreaks may occur among young dogs kept in kennels. Fatalities seldom occur in dogs older than 2 years of age. Vaccination has greatly reduced the frequency of the disease, and nowadays it is rare in many countries where it used to be endemic. Although the clinical symptoms, gross and histopathological findings of fatal cases are usually rather characteristic (Kelly, 1993), confirmation of the presence of CAV-1 is necessary for exact diagnosis. Isolation of CAV-1 on MDCK or vascular epithelial cells is often difficult and may require several blind passages before the cytopathic effect becomes obvious.

In the present work, a PCR method is described, which might serve as a useful alternative to virus isolation in the rapid confirmation of the diagnosis of infectious canine hepatitis.

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Materials and methods

Case history and clinical observations. Case 1. A four-month-old male Caucasian shepherd dog was submitted for *post mortem* examination to the Veterinary Institute of Debrecen, in the spring of 1995. The puppy had been vaccinated against canine parvovirus and canine distemper virus only. The first clinical symptoms of vomiting, high fever, and signs of abdominal pain appeared 2 days earlier. The puppy was found dead on the third morning.

Gross and histopathological examinations. Gross and histological examinations, including light microscopic examination of haematoxylin and eosin stained sections of the liver, were carried out at the Veterinary Institute of Debrecen. Organ samples taken from the liver were stored at –20 °C before being subjected to PCR.

Case 2. A similar case had been examined at the Central Veterinary Institute, Budapest, in 1992. Organ samples taken from the liver of the affected dog had been passed to the Department of Epizootiology and Microbiology, University of Veterinary Science, Budapest, where an adenovirus was isolated on MDCK cell line. The isolate was identified as CAV-1 by virus neutralization test with hyperimmune serum kindly provided by Professor L. E. Carmichael, Cornell University, Ithaca, USA. The isolated virus strain and liver specimen were kept frozen at –20 °C for over three years. These samples were used as specific positive controls.

Sample preparation. In order to expose the viral DNA we used two different procedures. The first one (Procedure A) was a standard DNA purification method including Proteinase K digestion, phenol/chloroform extraction, and alcohol precipitation steps (Benkő et al., 1988; Benkő, 1990). The other method (Procedure B) consisted of the following simple steps: approximately one gram of liver specimen was homogenized and diluted to 1:10 in PBS. After three cycles of freezing at –20 °C and thawing at room temperature, the sample was boiled for 10 min. One ml of the solution was transferred into an Eppendorf tube and was spun in a microcentrifuge at maximum speed (app. 13,000 rpm) for 5 min. An aliquot of 2 µl of the supernatant was used in the PCR mixture as target DNA solution.

Controls. Purified DNA of human adenovirus type 5 (HAV-5) served as positive control, while a liver sample taken from an uninfected dog carcass and treated as described above was used as negative control.

Primers. The sequence of the primers (HexAdB and HexAdJ) was described before (Kiss et al., 1996). The primers were synthesized on a Gene Assembler Special oligonucleotide synthesizer (Pharmacia LKB).

PCR. The amplification was carried out in a reaction mixture of 50 µl volume containing 5 µl 10 × reaction buffer (500 mM KCl, 15 mM $MgCl_2$, and 100 mM Tris-HCl, pH 9.0), 50 pmol of each primer, 1 U of thermostable Taq DNA polymerase, 300 µM of each of the four deoxynucleoside triphosphates (dATP, dCTP, dGTP, and dTTP; Pharmacia LKB), and 2 µl of sample solution. The "Step-Cycle" program of the DNA Thermal Cycler machine (Perkin-Elmer Cetus Instruments) was set to denature the DNA at 94 °C for 30 sec, to anneal the primers at 55 °C for 30 sec, and to extend the sequence at 72 °C for 30 sec, for a total of 35 cycles.

Detection of PCR products. The amplification products were detected by agarose gel electrophoresis. An aliquot of 8 µl from each reaction was run on 1.5% agarose gels containing ethidium bromide (5 µg/ml) at a constant voltage of 110 V for 45 min. For molecule weight marker λ phage DNA digested with *Pst*I enzyme was used. The gels were placed on a UV transilluminator and photographed on Polaroid 667 films.

Results

Gross and histopathological examinations. Necropsy of the Caucasian shepherd dog revealed an excess of bloody fluid in the abdomen, petechial haemorrhages and slight jaundice on the serous membranes. The liver was enlarged, turgid, and friable. The wall of the gallbladder was oedematous. Histological examination of the liver showed haemorrhages, and hepatic necrosis. There were some large, amphophilic intranuclear inclusions in the hepatic parenchymal cells indicating the presence of CAV-1. Similar gross and histopathological changes were seen in the dog of Case 2.

PCR. Figure 1 shows an agarose gel with the PCR products from positive control (HAV-5 DNA and Case 2) samples. The amount of the amplified product from CAV-1 appears to be less than that from HAV-5. Apart from this, it can clearly be seen that the size of the amplified DNA fragment corresponds to the size of the amplified segment of HAV-5.

Figure 2 shows the result of the PCR amplification of the tissue homogenate samples. In this experiment we applied the simple method (Procedure B) to expose the viral DNA, and found it satisfactory for our purpose. As can be seen in Fig. 1 (Lanes 6 and 7), there was only minor difference in the intensity of the two bands obtained from the same sample pre-treated in the two different ways. Obviously, the DNA concentration in the extracted sample can be much higher, but since it is contaminated with chromosomal DNA of cellular origin, if the sample is used undiluted, the performance of the reaction can be considerably diminished.

Discussion

PCR is a recently described *in vitro* method for primer-directed enzymatic amplification of specific target DNA sequences (Saiki, 1989). It has gained wide popularity in different fields of biology from basic research to routine diagnostics. In the developed countries, PCR has become an important part of medical diagnostic methods, and will certainly be introduced into certain fields of veterinary routine diagnostics as well (Belák and Ballagi-Pordány, 1993). When used with proper caution, PCR can provide an invaluable help in the rapid identification of specific pathogens which are difficult to grow.

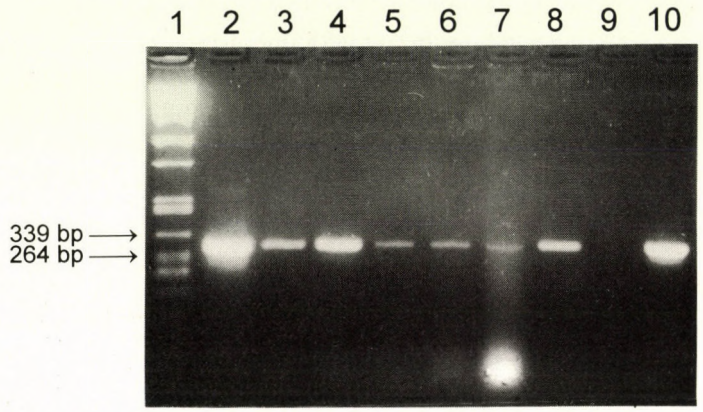


Fig. 1. Agarose gel with PCR amplification products from samples pre-treated by different procedures. Lane 1: molecule weight standard: λ phage DNA digested with *Pst*I enzyme; Lanes 2 and 10: positive control: HAV-5 DNA; Lanes 3–5: tissue culture fluid of the third passage of the isolated CAV-1 strain, treated either by procedure B (see in the text, Lane 3), or by procedure A (see in the text, Lanes 4 and 5). Lane 5 contains the PCR product from the same sample as Lane 4, but diluted 100 times prior to the reaction; Lanes 6–8: liver tissue homogenate from Case 2, treated either by procedure B (Lane 6), or by procedure A (Lanes 7–8). Lane 8: same as Lane 7, but the sample was 100 times diluted prior to the PCR; Lane 9: negative control: tissue homogenate (treated by procedure B) from the liver of an uninfected dog

In the present study, the performance of PCR was tested in detecting the presence of canine adenovirus type 1 in clinical samples. The applied primers were originally designed and tested for the detection of human adenoviruses (Allard et al., 1990), then with some modification for bovine, ovine, and porcine adenoviruses (Kiss et al., 1996). We have now demonstrated that CAV-1 might also be efficiently detected by these primers either in tissue culture (especially usefully in early passages of attempted virus isolation) or in clinical samples.

The advantage of PCR over virus isolation lays in its rapidity and simplicity. Successful amplification of certain segments of CAV-1 and CAV-2 DNA from inoculated cell culture supernatant without any chemical pre-treatment has recently been reported (Harasawa et al., 1994). We have also found that even a very simple treatment of the organ samples and tissue homogenates is sufficient for obtaining fast and reliable results. It might even be advisable to avoid the more labour-effective DNA extraction procedure, since co-precipitated chromosomal DNA (and other impurities of cellular origin) or even just a too high concentration of target DNA seem to hamper the amplification reaction (Fig. 2, lanes 7 and 8). We have not intended, however, to address the problems of quantitative conditions in the framework of this study.

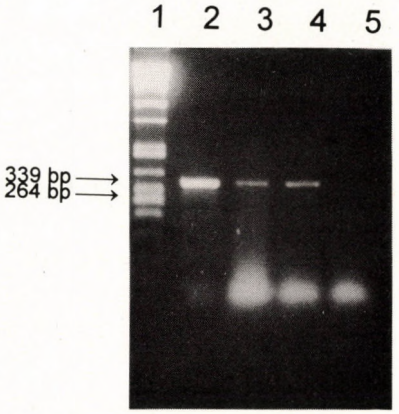


Fig. 2. Agarose gel with PCR amplification products from tissue homogenate samples (procedure B) as listed below. Lane 1: molecular weight marker: λ phage DNA digested with *Pst*I enzyme; Lane 2: positive control: HAV-5 DNA; Lane 3: specific positive control: unextracted liver tissue homogenate from Case 2. Lane 4: unextracted liver tissue homogenate from Case 1; Lane 5: negative control: unextracted liver tissue homogenate from an uninfected dog

Because of the widely used preventive vaccination, infectious canine hepatitis has by now become a rare disease in Hungary. Consequently, there are fewer fatalities to be seen. Since the preparation of this manuscript, a suspected case of adenovirus-induced canine hepatitis has turned up. The PCR performed on the very day of the postmortem examination, however, gave negative result. The histopathological examination carried out on the subsequent day also failed to demonstrate the characteristic inclusion bodies, implying that hepatitis in that dog was not of adenoviral origin. We believe that the PCR method described here might be useful even in the exclusion of the presence of adenoviral DNA.

Acknowledgements

The authors are indebted to Professor L. E. Carmichael, Cornell University, Ithaca, for providing the positive sera for the virus neutralization test. Part of this work was supported by the National Research Fund of Hungary, grants OTKA I/3 1984, I/7 T016882, I/8 T021060, and A312. István Kiss and Katalin Matiz are enrolled Ph.D. students of the University of Veterinary Science, Budapest, Hungary.

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BOOK REVIEWS

Johannes KAUFMANN: ***Parasitic Infections of Domestic Animals. A Diagnostic Manual.*** Birkhäuser Verlag, Basel, Boston and Berlin, 1996. 423 pages, 718 illustrations (nearly half of them coloured), 28 tables. Price: sFr. 68.

The inventive outline of "Diagnose und Therapie der Parasiten von Haus-, Nutz- und Heimtieren" published by H. Mehlhorn, D. Düwel and W. Raether first in 1986 and updated in 1993 was highly appreciated by German speaking/reading parasitologists. The failure of the book to attain a wider use is certainly due to the language barrier. Now, this book of similar structure by J. Kaufmann seems apt to fill this gap in English-speaking territories. Although it is not a textbook, an enormous amount of information, pertinent mainly to the diagnosis and control of the cosmopolitan parasites and parasitoses with particular emphasis to those occurring in the African continent, is included both through the text and the rich, excellent illustrations. All this makes the manual a pragmatic aid particularly in diagnostic and research laboratories.

Of the seven chapters the book comprises, the first deals with methods widely used in every conventional parasitological laboratory and also with immunological and recently introduced molecular biological techniques (the latter compiled by D. Dobbelaere and I. Roditi). The subsequent chapters include parasites of cattle, those of sheep and goats, horses and donkeys, dromedaries, swine, and poultry. Unfortunately, a chapter devoted to carnivores (dogs and cats) or to rabbits is missing. The book is supplemented with a bibliography, a list of further reading and an index. The parasites (in the order of protozoa, helminths, arthropods) are arranged according to the host's organs as stages can be detected in the gut and faeces, blood and circulatory system, urogenital system, internal organs (locomotor system, liver, respiratory system), abdominal cavity, central nervous system, and on the body surface. The author, head of Diagnostic Section of the Institute of Parasitology at the University of Berne, having formerly worked in the field laboratory of the International Trypanotolerance Centre of Gambia and thus being very well aware of the differential diagnostic concern of blood parasites with several rickettsial organisms (*Anaplasma, Cowdria, Ehrlichia, Eperythrozoon*), added these "parasite like" agents to the real parasites as they are encountered during routine diagnostic work. A swift orientation in the copious body of information is helped with each specific infection by reasonably consistent, stereotypic subheadings such as location, hosts, species description, geographic distribution, symptoms, significance, diagnosis, therapy, prophylaxis, occasional taxonomic remarks, useful cross-references among the species, saving space and diminishing redundancies, and also by the careful colour-coded layout of the manual.

The excellent illustrations showing stages of parasites either in nice colour or black and white, instructive drawings of morphology and life cycles, macroscopic and microscopic photographs, tables containing differential characteristics of related species or drugs are concise and invaluable sources of information.

The hoped next edition might be improved by considering minor inaccuracies which easily occur in a book of this scope. For instance, among the almost perfect coloured illustrations, Fig. 30 hardly enables identification of the egg of *Fasciola hepatica* by its present colour; doramectin, a broad-spectrum endectocide drug should not only be mentioned among compounds to control external parasites of cattle; *Buxtonella* "oocysts" might be replaced by "cysts"; *Eimeria mivati* is no longer a valid species; parasites of avian hosts should not be restricted almost to chicken, and might include e.g. turkey and goose coccidiosis, amidostomosis, etc., although they are of secondary importance in most parts of the world. Inclusion of the parasitic infections of rabbits and particularly those of dogs and cats would certainly be appreciated by the prospective users of the manual.

The book is nicely printed, and has an appealing format. It is another contemporary parasitological manual which will become an indispensable and widely used aid on the bookshelves of universities, veterinary diagnostic and research laboratories, meat inspectors, veterinary practitioners and students.

István Varga

Charles O. Thoen and *James H. Steele* (eds): ***Mycobacterium bovis* Infection in Animals and Humans.** Iowa State University Press, Ames, 1995. 374 pages, illustrated, hardcover. Price: $54.95. ISBN 0-8138-2120-7

The English-language manual entitled "*Mycobacterium bovis* Infection in Animals and Humans", written and edited by *Charles O. Thoen*, DVM, PhD, Professor of Microbiology, Immunology, and Preventive Medicine, College of Veterinary Medicine, Iowa State University, and *James H. Steele*, DVM, MPH, Professor Emeritus, Environmental Health, The University of Texas-Houston, School of Public Health, Center for Infectious Diseases, was published by Iowa State University Press in 1995. The text contains introductory contributions by representatives of international organizations such as the International Union Against Tuberculosis and Lung Diseases (IUATLD), the World Health Organization (WHO), and the Food and Agriculture Organization (FAO), the Pan-American Health Organization, and the Office International des Epizooties. Thirty-one international experts contribute to this report on occurrence and eradication of *M. bovis* around the world.

The book, which runs to 374 pages, consists of two parts. Part 1 was written and edited by Professor Charles O. Thoen. This 166-page first part comprises the following 13 topics: (1) Pathogenesis of *Mycobacterium bovis*. (2) Molecular techniques: Applications in epidemiologic studies. (3) Human aspects of *Mycobacterium bovis* infection. (4) *Mycobacterium bovis* BCG infection in humans. (5) Laboratory diagnosis of bovine

tuberculosis. (6) Tuberculin production. (7) Tuberculin skin tests: Sensitivity and specificity. (8) Tuberculosis in captive wild animals. (9) Vaccination: Potential applications. (10) Bovine tuberculosis eradication: A program in the United States of America. (11) Tuberculosis in a large confined dairy herd: Approach to eliminations. (12) *Mycobacterium bovis* in captive Cervidae: An eradication program. (13) Control of bovine tuberculosis in developing countries.

The chapters dealing with the 13 topics were written by 22 co-authors. These topics give evidence of a high-level theoretical synthesis and, due to the brilliant editing, the theoretical statements are in perfect harmony.

Part 2, edited by Professor James H. Steele, presents regional and country status reports of *Mycobacterium bovis* infection for a total of 98 countries, grouped by geographical regions (countries of South, Central and North America; the Caribbean; countries of Europe and Africa; Central Asia, Southeast Asia and India; Oceania and the Far East). The data relating to the different countries are rendered convincingly authentic by reports presented by specialists from the countries concerned.

The book is concisely written and has a clear structure. All chapters are complemented by a reference list. Reader orientation is facilitated by an alphabetical index.

Besides being an up-to-date reference tool, this book is a gap-filling and invaluable aid to both theoretical and practical specialists, researchers, specialists working in diagnostic laboratories, clinicians, epidemiologists, veterinarians and zootechnicians all over the world.

Béla Körmendy

Roy MACK, Bettina MIKHAIL and Michel MIKHAIL: ***Wörterbuch der Veterinärmedizin und Biowissenschaften/Dictionary of Veterinary Medicine and Biosciences.*** German–English · English–German. With a quadrilingual appendix: Latin–German–English–French. Second Edition. Blackwell Wissenschafts–Verlag, Berlin–Wien, 1996. 823 pages. Price: DM 168. ISBN 3-8263-3055-2

The Second Edition of the Dictionary of Veterinary Medicine has been completely revised, and nearly 10,000 new entries have been added. The original author, Roy Mack, has been joined by Bettina Mikhail and Michel Mikhail, who have contributed their own expertise to the dictionary. The dictionary is intended as a reference tool for veterinarians, those engaged in the biological sciences, and translators. Veterinary drugs are included under their international generic names. An appendix lists Latin terms in anatomy, medicine and zoology, with equivalents in German, English and French.

The second edition of this dictionary has expanded to about 20,000 entries to include new terms and (for the first time) names of medicines, also to extend the coverage of anatomical and animal names. The aim is to supplement the standard German-English general dictionaries with technical terms in the fields of anatomy, microbiology,

physiology, parasitology, pathology, pharmacology, toxicology and zootechny, with special reference to domestic animals and their diseases.

Animals: Apart from the domestic animals, the common wild animals of Europe, those present in zoos, and exotic pets are included. Invertebrates are confined to pets of livestock and vectors of disease. Latin names of animals are contained in the separate list of Latin terms (Part III).

Medicines: are included under nonproprietary names, preferably an International Nonproprietary Name of the World Health Organization. Proprietary names of drugs are generally outside the scope of this dictionary, except for a few instances where the proprietary name is widely used. Pharmacological groups and many other individual compounds of pharmacological interest are included.

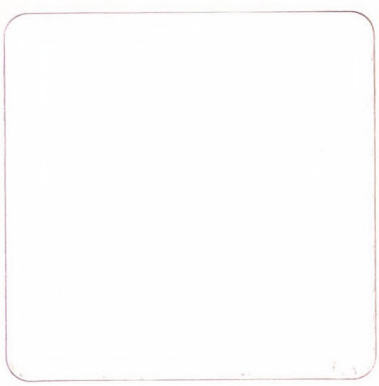
Anatomy: The nomenclature of *Nomina Anatomica Veterinaria* (fourth edition) and *Nomina Histologica* (second edition) has been followed. 6500 Latin terms are listed separately in Part III, with their German, English and French equivalents.

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NOTES FOR CONTRIBUTORS

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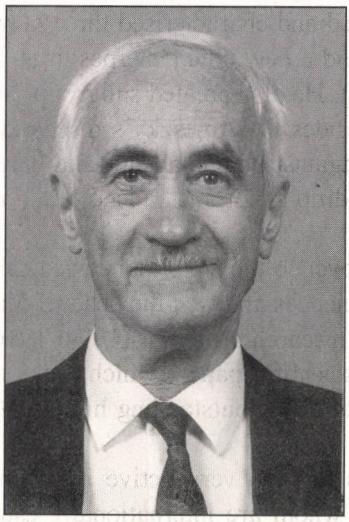
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Adorján Bartha (1923–1996)

The Hungarian Academy of Sciences and its Veterinary Medical Research Institute, Budapest announce with the deepest sorrow that **Adorján Bartha**, Corresponding Member of the Hungarian Academy of Sciences, former Director of the Veterinary Medical Research Institute of the Hungarian Academy of Sciences, Titular Professor of the University of Veterinary Science, Budapest, member of several international scientific societies, holder of the Széchenyi Prize (1995) as well as several international and Hungarian awards and commemorative medals, died on 27 May, 1996, at the age of 73. His funeral was held in the Budapest Farkasréti Cemetery on 11 June, 1996.

Adorján Bartha was born on 12 December 1923 in Porcsalma, Hungary. He attended the primary and secondary school in Debrecen and graduated from the College of Veterinary Medicine, Budapest in 1949. He received his PhD degree at the same prestigious college in 1959, where he worked at the Department of Epidemiology and Microbiology (led by Professor Rezső Manninger) first as assistant professor (1949–1956), then as lecturer (1957–1967), and as associate professor (1968–1974). During these years he established the first modern veterinary virology laboratory in Hungary (with the late Professor Tamás Szent-Iványi). Here he developed the very first fully attenuated Aujeszky's disease virus vaccine strain (K61), and started the career of the Bartha-vaccine used in several countries

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even today. He also isolated and characterised three new types of bovine adenovirus (BAV-4, -5, and -8) and a new bovine herpesvirus (BHV-4, that he originally named Movar herpesvirus). He also created subgroup 2 of bovine adenoviruses as a new taxonomic unit. Besides the Aujeszky's disease vaccine he developed several other virus vaccines against bovine adeno- and herpesviruses, equine herpesvirus infections, the "egg drop syndrome" adenovirus, and canine parvovirus diseases.

In 1974 he moved over to the Veterinary Medical Research Institute of the Hungarian Academy of Sciences and was the head of Virology (1975–1990), then director (1990–1994) and research professor (1994–1996) of that institute. He has authored more than 150 scientific papers which have so far been cited approximately 1400 times. Several of his outstanding highly cited papers were published in our journal.

In both institutions he was very active in teaching and in working with young scholars, many of whom are internationally established scientists abroad and in Hungary. Several young scholars from other countries were fortunate enough to spend months (or years) in his laboratory. They all regard him as master of their art.

His dedicated work was greatly acknowledged by international societies and organisations. Among others, he was an officer of the WHO/FAO Comparative Virology Programme, president of the WHO/FAO Adenovirus Working Group and member of the ICTV Adenovirus Study Group. He was honorary distinguished member of the European Society for Veterinary Virology, winner of several Hungarian and international medals, and member of the editorial or advisory boards of international journals, among others the *Acta Veterinaria Hungarica*.

With his death Hungarian veterinary science has lost an outstanding scholar, a distinguished scientist who is mourned by the entire veterinary profession and the agricultural community, his fellow academicians, disciples, colleagues and friends both in Hungary and abroad.

His excellent scientific judgement and creative vision, his originality and intellectual inspiration will be missed so much. The rich spiritual heritage and the unique professional values that he represented will be preserved by us.

October 1996

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AGE-RELATED CHANGES IN SCROTAL CIRCUMFERENCE OF HUNGARIAN SIMMENTAL BULLS IN SELF-PERFORMANCE TEST

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Between 1989 and 1992, investigations were carried out on a total of 164 young Hungarian Simmental bulls of different age at central performance testing stations and on farms (field test) to measure the scrotal circumference of bulls and describe its relationships with age and body weight. An age-related increase was found in average scrotal circumference in the populations studied. Average scrotal circumference of Hungarian Simmental bulls was 17.86 cm at 5 months of age and 37.06–37.72 cm at 13–14 months of age. The coefficient of correlation between age and scrotal circumference was between r = 0.31 and r = 0.40, while that between scrotal circumference and body weight varied between r = 0.35 and r = 0.59. The partial regression coefficients for age and body weight showed values of r = 0.003–0.063 and r = 0.017–0.032, respectively. The multiple coefficient of correlation was between R = 0.47 and R = 0.59.

Key words: Breeding bulls, body weight, development, scrotal circumference

The measurement and evaluation of scotal circumference serve as a method for the preselection of breeding bulls in several countries (Australia, Canada, France, U.S.A.). The scrotal circumference values of yearling bulls may provide the following information.

(i) A larger scrotal circumference improves certain parameters of semen quality such as motility, percentage of live sperm cells, number of sperm cells, and density of the semen (rg = 0.09–0.58), while the number of abnormal sperm cells decreases (rg = –0.07 to –0.52) (Lunstra, 1986; Brinks, 1987; Nwakalor and Enzima, 1989). On the contrary, some bulls with a scrotum of small size are likely to produce high quality semen (Amstutz, 1987).

(ii) A close negative relationship (r = –0.9) was found between scrotal circumference and early sexual maturity in eight different beef and dual-purpose breeds (Brinks, 1987). Smith et al. (1989) found a close negative correlation

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($r = -0.796$) between scrotal circumference of the sires and early sexual maturity of their daughters. Thus, as the daughters and half-sisters of the sires become sexually mature earlier, they can be bred earlier, too.

There are few data in the literature about the development of scrotal circumference and testicle measurements of Hungarian bull populations (Balika et al., 1976; Asem, 1980; Becze, 1983; Varga, 1990; Tőzsér, 1991; Gábor et al., 1993, 1995). The development of the scrotum of yearling bulls is evaluated according to the following classification system, as a function of body weight: *very good:* scrotal circumference reaches or exceeds the standard value; *good:* scrotal circumference is smaller than the standard value but the difference is not more than 2 cm; *undesirable:* scrotal circumference is more than 2 cm smaller than the standard value.

International research has been focusing on 12–14 months old or even older bulls to analyse the effect of age and body weight on scrotal circumference. The correlation coefficients varied between wide extremes in both respects ($r = 0.2$ to 0.8).

The main objective of the present study was to measure the average scrotal circumference of Hungarian Simmental bulls of different age in the field and at central performance testing stations. The second goal was to describe the relationship between scrotal circumference and age on the one hand and scrotal circumference and body weight on the other.

Materials and methods

The investigations were carried out on several farms and at central self-performance testing stations between 1989 and 1992. Hungarian Simmental ($n = 232$) bulls of different age were included in the study.

The scrotal circumference of young bulls (sire candidates) was measured, with one exception, at the end of the test, at the widest part of the scrotum (Taylor, 1984).

In the evaluation of results some characteristics of the Hungarian self-performance testing system must be taken into consideration. At the central performance testing stations the bulls are kept individually under intensive nutrition, while bulls in the field testing stations are kept in groups of 15 to 20 animals and fed mainly bulk feeds (less intensive technology). The other problem is that A. I. bulls are different in consecutive years. For that reason, the results obtained for bulls kept at central and at field testing stations have to be evaluated separately in each year.

To describe the relationships among scrotal circumference, age and body weight, the methods of one-variable and two-variable linear analysis of regression were used (Snedecor and Cochran, 1976).

Results

The average values of age, body weight and scrotal circumference of the investigated populations of Hungarian Simmental bulls are summarized in Table 1.

Table 1

Age, body weight and scrotal circumference of young Hungarian Simmental bulls at different testing stations and in different years (mean ± S.D.)

Herd and system of performance test	Year	Number of bulls	Age (days)	Body weight (kg)	Scrotal circumference (cm)
I. CPT*	1991	80	397 ± 21.57	560 ± 72.47	37.72 ± 2.54
II. FT**	1989	17	433 ± 15.96	598 ± 45.60	37.06 ± 3.03
III. FT	1992	67	144 ± 20.35	196 ± 29.07	17.86 ± 1.64

*CPT = central self-performance testing station; **FT = field test

The correlations between scrotal circumference and age and those between scrotal circumference and body weight were different in the populations tested, and they also depended on the year of investigation. The correlation was very close, as calculated for the whole population irrespective of the year of investigation and the testing station (Table 2).

Table 2

Correlation coefficients (r) between scrotal circumference and age as well as between scrotal circumference and body weight of bulls

Herds	Number of bulls	Age	Body weight
I	80	0.31*	0.52**
II	17	0.40	0.35
III	67	0.34*	0.59**

Levels of significance: *$P < 0.01$, **$P < 0.001$

The multiple coefficient of correlation among the measured parameters varied within wide limits (R = 0.47 to 0.59), in the same way as the partial coefficients of correlation between scrotal circumference and age, as well as scrotal circumference and body weight (Table 3).

Table 3

Multiple coefficients of correlation among scrotal circumference, age and body weight in different populations of the Hungarian Simmental breed

Herds	n	Partial regression coefficients, b		Partial correlation coefficients, r		Multiple correlation coefficients	Determination coefficients
		Age	Body weight	Age	Body weight	R	R^2 %
I	80	0.024	0.017	0.232*	0.491**	0.56	31
II	17	0.063	0.018	0.342	0.277	0.47	22
III	67	0.003	0.032	0.138	0.516**	0.59**	35

Levels of significance: *P < 0.01, **P < 0.001

Discussion

Data available in Hungary on the scrotal circumference of young breeding bull candidates are rather scanty. Therefore, any result obtained in this respect can be a useful contribution to the elaboration of standards for the scrotal circumference of different breeds.

The scrotal circumference data taken at the end of the self-performance test were consistent with international findings in the Hungarian Simmental breed.

Measurement of the scrotum at the beginning of the performance test is justified by the close correlation (r = 0.6) between consecutive circumference data, which makes it possible to cull bulls with underdeveloped testicles before the beginning of the self-performance test.

The correlation coefficients suggest that the body weight and the age of young bulls have a similar effect on scrotal circumference. The relevant figures obtained are r = 0.35–0.59 (body weight) and r = 0.31–0.40 (age).

The determination coefficients were found to vary between wide extremes (R^2 % = 22 to 35). Therefore, when evaluating the reproductive status of bulls, only animals of about the same age (with an age difference of less than 60 days) and of similar body weight should be compared.

The following conclusions can be drawn from this study:

(1) To judge the reproductive status, the scrotal circumference of breeding bull candidates can be measured both in the field and at the central performance testing station.

(2) Body weight and age exert a similar effect on the development of scrotal circumference.

(3) Results of the multivariate analysis of variance emphasize that only animals of the same age and body weight are comparable from the point of view of reproductive status.

Acknowledgement

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EVALUATION OF THE GnRH TEST RESULTS OF BEEF-TYPE BREEDING BULL CANDIDATES

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(Received March 12, 1996; accepted July 10, 1996)

The GnRH test results of Hungarian Fleckvieh and Charolais breeding bull candidates were evaluated by two different methods (selection based on previously fixed standard data and on standard deviation, respectively) in two breeding herds. The Leydig's cell response of the testicles to a central signal after the injection of a single dose of 100 μg exogenous GnRH analogue (Ovurelin inj. ad us. vet., Reanal Co. Ltd., Budapest) was evaluated. The two different selection methods used for evaluating the results of the GnRH test brought different results: on the basis of phenotypic value three, while according to the absolute value seven Hungarian Fleckvieh individuals were selected as excellent. For the evaluation of the results of GnRH treatment, the phenotypic deviation value should be used in the practice because of its easy interpretation. The relative phenotypic values changed between 84.4 and 136.6 in the Hungarian Fleckvieh and between 64.7 and 179.4 in the Charolais breed.

Key words: Bull, GnRH test, selection, testosterone

The selection of breeding bull candidates is based on several production and phenotypic criteria; however, the reproductive status of such bulls has not been investigated regularly. There are some proposals for using the so-called GnRH test, based on induced testosterone production of the testicles as a criterion in the selection of breeding bulls. The main objective of this study was to evaluate the results of the GnRH test in two beef bull populations.

The preliminary selection of young beef bulls is based on phenotypic markers such as health status, condition score and meat type but does not extend to the prediction of fertility. Thus, evaluation of the andrological status of young bulls during performance testing seems to be of basic importance.

Several methods have been proposed which take into consideration the reproductive or andrological status of young bulls as selection criteria (Post et al., 1987*b*). Most of the methods are based on scrotal circumference, sperm qualification or an evaluation of Leydig's cell activity of the testicles, i.e. the GnRH test which shows the responsiveness of cells to a central signal.

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When interpreting the GnRH test, certain criteria must be taken into consideration as they may affect the outcome of the test and lead to false results. The biological half-life of oligopeptides such as GnRH is very short. For that reason, the route of application (intramuscular or intravenous) is very important because of the different response interval (Tannen and Convey, 1977; Schambacher and Echternkamp, 1978; Bass et al., 1982). The strength of the signal is also important in the evaluation of response, rendering the dose of the GnRH preparation as the second criterion (Post et al., 1987*a*). There are several proposals suggesting that the dose should be calculated on the basis of the bulls' actual body weight. The third criterion is the length of the response interval. Preliminary results and practical observations indicate values between 15 and 120 min. The oligopeptide GnRH acts as hapten; therefore, the time sequence of treatment is the fourth criterion because of a possible down-regulation phenomenon as has been found earlier (Mongkonpunya et al., 1975; Tannen and Convey, 1977; Oltner et al., 1979; Malak et al., 1985).

Another important part of the evaluation is the determination of testosterone. There are several different methods for the measurement of steroids, the most recent being immunoassay procedures. Of them, enzyme- and radioimmunoassay methods are preferred, which differ in the use of tracer (horse-radish peroxidase, ^{3}H or ^{125}I) and the procedure (direct or requiring extraction). The differences are measurable and may sometimes lead to false results (Mézes et al., 1993).

Several methods are available for the evaluation and use of the GnRH test for the ranking and selection of young breeding bulls. The first method of ranking is based on the testosterone level of blood serum after a single dose of GnRH analogue. The second method ranks the bulls on the basis of their serum testosterone level after a single dose of GnRH analogue and compared to previously established standard values (Schambacher, 1979; Veeramachaneni et al., 1986). The third method ranks the bulls on the basis of their serum testosterone level before and after the same GnRH dose (Wekerle et al., 1989).

The purpose of the present study was to compare the two basic methods of evaluation, selection based on previously fixed standard data, and selection based on phenotypic standard deviation.

Materials and methods

Young breeding bulls from two Charolais and Hungarian Fleckvieh breeding farms (farm A: n = 10; farm B; n = 40) were used. The young bulls were kept in loose housing system, in small groups, and were fed a diet based on corn silage and concentrate.

The following data were measured and evaluated during the study: liveweight; scrotal circumference, as recommended by Taylor (1984); the Leydig's cell response of the testicles to a central signal (GnRH response): measurement of serum testosterone level before and 120 min after a single dose of 100 μg exogenous GnRH analogue (Ovurelin inj. ad us. vet., Reanal Co. Ltd., Budapest).

The response was considered "optimal" if the serum testosterone level was ≥ 34.7 nmol/l 120 min after a single intramuscular dose of GnRH (Schambacher, 1979; Veeramachaneni et al., 1986).

The quantitative analysis of testosterone was carried out by direct ^{125}I radioimmunoassay with matrix (steroid-free bull serum) correction (Institute of Isotopes Ltd., Budapest). The assay parameters were as follows: T/Bo: 0.39, NSB/Bo: 0.36, intra-assay CV%: 5.6, inter-assay CV%: 9.1.

The phenotypic value of individual bulls based on the hormone levels was calculated as follows:

$$Pvi = 100 + [20 \times (TAi - ATA)] : SD,$$

where: Pvi = Phenotypic value of any bull as calculated on the basis of the testosterone level of the blood serum after GnRH treatment (points);

Tai = Testosterone content of the blood serum of any bull after GnRH treatment (nmol/l);

ATA = Average testosterone content of the blood serum after GnRH treatment (nmol/l);

SD = Standard deviation of average testosterone content of the blood serum after GnRH treatment within the population.

The calculation was based upon the above equation and 1 SD value means 20 points. Based upon the score achieved, the bulls were classified as follows: excellent: breeding value score = 140–161; good: breeding value score = 121–140; acceptable: breeding value score = 101–120; poor: breeding value score < 100.

Results and discussion

Data (mean ± SD) on the age, liveweight and scrotal circumference of Hungarian Fleckvieh and Charolais breeding bulls, as well as their serum testosterone concentrations before and after GnRH treatment are shown in Table 1.

Table 1

Age, liveweight, scrotal circumference and serum testosterone (T) level of breeding bulls before and after GnRH treatment (mean ± SD)

Parameters	Farm A (Hungarian Fleckvieh)	Farm B (Charolais)
Number of bulls	10	40
Age (day)	350.00 ± 18.83	463.00 ± 37.81
Liveweight (kg)	557.20 ± 26.59	602.50 ± 54.23
Scrotal circumference (cm)	24.17 ± 6.63	22.40 ± 6.36
T concentration of blood serum before treatment (nmol/l)	24.17 ± 6.63	22.40 ± 6.36
T concentration of blood serum after treatment (nmol/l)	45.97 ± 16.71	28.38 ± 7.75

Average scrotal circumference of bulls of both breeds (Hungarian Fleckvieh: 35.8 cm, Charolais: 37.8 cm) corresponded to the animals' age as well as to data reported in the international literature (Coulter, 1986). The so-called basic testosterone concentration of the serum before GnRH treatment was similar in the two breeds (Hungarian Fleckvieh: 24.17 nmol/l; Charolais: 22.40 nmol/l). However, testosterone concentrations measured after GnRH treatment were different in the two breeds (Hungarian Fleckvieh: 45.97 nmol/l, Charolais: 28.38 nmol/l). This could be explained by age and breed differences and individual variation. Within each of the two breeds, GnRH resulted in testosterone concentrations which were significantly ($P < 0.05$) higher than the base value. Serum testosterone concentrations found after GnRH treatment are shown in Table 2 according to two different selection criteria (phenotypic and absolute value).

On the basis of the phenotypic value only three, while according to the absolute value seven individuals were selected from the 10 Hungarian Fleckvieh bulls examined in farm A.

Statistical analysis of the differences between group averages was prevented by the low number of individuals and the relatively high values of standard deviation. Average serum testosterone concentration after GnRH treatment was higher for the three bulls selected on the basis of the phenotypic value than in the other group. In contrast to the results obtained for the Hungarian Fleckvieh, the number of bulls selected on the basis of the phenotypic value was higher ($n = 17$) in the Charolais breed; however, average testosterone concentration measured after GnRH treatment was by 6.86 nmol/l lower in this group than in animals selected on the basis of the absolute value.

Table 2

Serum testosterone (T) concentration after GnRH treatment using different selection criteria

	Farm A (Hungarian Fleckvieh)		Farm B (Charolais)	
Selection criterion	PV > 100	T > 34.7 nmol/l	PV > 100	T > 34.7 nmol/l
Number of individuals	3	7	17	6
Average	66.10	51.36	34.58	41.44
SD ±	17.42	17.47	7.36	8.85
Minimum	45.99	35.67	28.76	35.55
Maximum	76.53	76.53	59.16	59.16

PV = phenotypic value regarding serum testosterone concentration

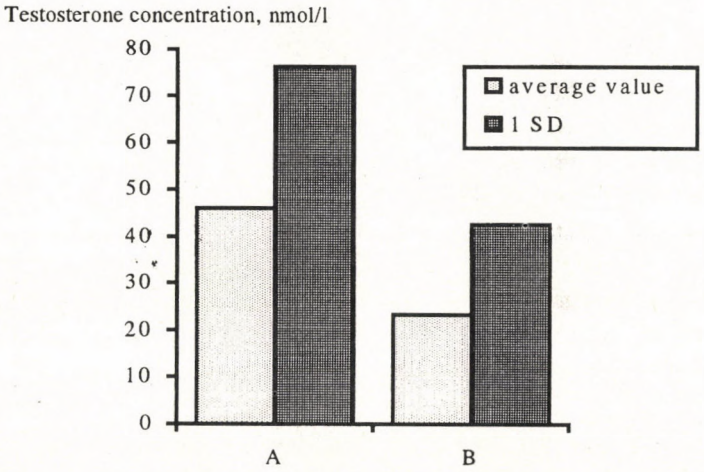


Fig. 1. Serum testosterone concentration of bulls exceeds the value of standard deviation of the phenotypic value after GnRH treatment; A = Hungarian Flechvieh (n = 2), B = Charolais (n = 5)

It is well known that only those animals can be stated to have an improving effect whose improving effect concerning a given trait reaches or exceeds the deviation value for that population. Evaluation of the results of the GnRH test by comparing them to the absolute value is impossible. In contrast, evaluation on the basis of the relative phenotypic value enable an exact judgement of superior performance. For the Hungarian Fleckvieh and Charolais breeds, the relative phenotypic values were between 84.4–136.6 and 64.7–179.4, respectively. The testo-

sterone concentrations corresponding to these values were: 32.94 to 76.53 nmol/l for the Hungarian Fleckvieh and 14.71 to 59.16 nmol/l for the Charolais. Using the relative phenotypic value, the average testosterone concentration of the serum after GnRH treatment was much higher in bulls which reached or exceeded the value of standard deviation of the phenotypic value (Fig. 1).

The results show that only a few individuals have an improving effect within the population studied.

The following conclusions can be drawn from the present work: The two different selection methods used for evaluating the results of GnRH treatment brought different results. Using the phenotypic and the absolute value three and seven Hungarian Fleckvieh bulls, respectively, were selected in farm A. For evaluating the results of GnRH treatment the phenotypic deviation value should be used because of its easy interpretation. The relative phenotypic value varied between 84.4 and 136.6 points in the Hungarian Fleckvieh and between 64.7 and 179.4 points in the Charolais.

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OCCURRENCE OF LISTERIA AND LISTERIOSIS IN HUNGARY*

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Listeriosis is a rare human disease in Hungary. The number of cases is slowly increasing. Only sporadic events have been observed but the occurrence of epidemic listeriosis may be supposed. The *Listeria monocytogenes* (in abbreviation: *L. m.*) transmitter role of food in human infections has not yet been verified. The epidemiological character of animal listeriosis is different. Healthy carriers can be found among both humans and animals. Foodstuffs of animal as well as plant origin may be contaminated with Listeria. When the processing technology and/or hygienic conditions are not satisfactory, these microorganisms can be detected in food factories and in final products of the food industry.

Key words: Listeria, human and animal listeriosis, surveillance, food, contamination, listeriosis in Hungary

Information concerning animal and human listeriosis in Hungary has been collected since the 'fifties but there has not been obligatory notification. Besides data on listeric patients and dead animals, the incidence of healthy human and animal excretors of Listeria has also been studied. The presence of Listeria in or on raw meats, meat products, raw milk, dairy products, cold dishes, salads, frozen foods and confectionery, as well as Listeria contamination of the environment and equipment in food factories have been investigated. The most important results are summarised in this paper.

Materials and methods

For cultivation and isolation of *Listeria* strains from samples, different media (modified Holman, UVM, Fraser, TNSA, LPM, Oxford, etc.) and methods (cold enrichment, oblique light technique) have been used (Ralovich, 1993). The number of listeriae in food samples (raw sausages, raw salamis, ice creams) was

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also examined by the MPN method. Five g samples of sausages, 10 g samples of salamis and 1 ml samples of ice creams were tested. Sterile swabs were used for taking samples from an about 100 cm^2 area on the outside or inside surface of tools, equipment, walls, drains, vessels, floors and other surfaces.

Results and discussion

Human listeriosis. As regards the number of healthy human Listeria carriers, from the results of earlier screening tests it seems that 2-3% of the population may excrete these microbes via the faeces in Hungary (Ralovich, 1991). The first bacteriologically verified human listeriosis case was diagnosed in 1965 (Rodler and Szenes, 1966). Since then, data connected with listeriosis cases have been collected voluntarily in our country. On the basis of this collection, *L. m.* infection was diagnosed in 200 patients between 1965 and 1994 (Table 1).

Table 1

Number of bacteriologically verified human listeriosis cases in Hungary between 1965 and 1994

Year	Number of cases	Year	Number of cases	Year	Number of cases
1965	1	1979	7	1988	13
1968	4	1980	1	1989	12
1969	4	1981	2	1990	12
1970	3	1982	6	1991	23
1971	2	1983	5	1992	20
1973	5	1984	2	1993	27
1975	3	1985	4	1994	16
1976	2	1986	9		
1978	4	1987	13	Total	200

The increase in the numbers of cases during the 'eighties can be explained by the fact that since then *L. m.* could be cultivated and identified in all the laboratories of the Public Health Service. In 1991, 1992 and particularly in 1993 the number of listeriosis cases was unusually high. It can be concluded that the incidence of human listeriosis is low in our country as compared with the international data. The mortality rate of listeric patients is high. From the data, it is possible that there was an epidemic in 1991–1993, although, since no epidemiological investigations were carried out, this cannot be confirmed. In order to obtain more

accurate information, it has been decided to set up an active listeriosis surveillance programme, using a data collection form. Foods have not yet been shown to transmit listeria.

Presence of Listeria strains in food samples. Rédei (cit. Ralovich, 1984) mentioned the isolation of virulent *L. m.* from creamy rice soup in 1960. Forray (cit. Ralovich, 1984) observed that Listeria strains could be isolated from the surface of raw pork suitable for human consumption in 1969. Systematic examination of food for Listeria began during the 'eighties. In contrast with this, there is no obligatory control examination of foods for *L. m.* except when requested by the authorities of an importing country. The results of examinations carried out by various investigators are presented in Tables 2–6.

Raw meats and raw meat products are most often contaminated with Listeria organisms. The counts obtained in a sample of 4 raw sausages were 540, 1100, 1600, and more than 1600 Listeria organisms per g, respectively, while those found in a sample of 3 raw smoked sausages were 0.0, 6.8 and 540 Listeria per g, respectively (Ralovich and Proksza, 1988). Out of 62 samples of salamis, 48 contained less than 0.3 *L. m.* per g, 8 had 0.3–0.4 *L. m.* per g, 4 samples 0.4–0.9 *L. m.* per g, and 2 samples 0.9–2.3 *L. m.* per g. The ripening process did not alter the number of Listeria-positive salami samples (Domján-Kovács, 1994).

Positive samples from cooked and semi-preserved products may mean that the treatment was insufficient or hygienic conditions after treatment in the shops were inadequate.

Raw milk and raw tank milk may contain Listeria as can be seen in Table 3. Dairy products may also be contaminated with these microbes. Pasteurisation of raw milk is obligatory at 76 °C for 37 sec in Hungary. This process is safe and inhibits transmission of listeriae by milk. In spite of this, samples of pasteurised chocolate milk from different small retail shops frequently proved positive for Listeria. Chocolate milk may therefore be a source of Listeria infection.

Our results concerning Listeria contamination of foodstuffs are similar to those in other countries.

From the data in Tables 4 and 5 it can be concluded that Listeria may be present not only in cold dishes, salads and frozen foods but also in confectionery.

In ice cream the bacterial counts were 10 *L. m.*/g in 1 sample, 100 *L. m.*/g in 3 samples, 1000 *L. m.*/g in 2 samples, and 10,000 *L. m.*/g in 1 sample (Szemenyei, 1994).

Listeriae in the food industry. The prevalence of Listeria strains in the environment of food factories has also been studied (Table 6).

Listeria strains including *L. m.* could be found in a wide variety of food industrial plants. After cleaning and disinfection the rate of positive samples de-

creased, so these activities proved to be quite effective against Listeria contamination. Following hygienic rules and HACCP principles is essential for the prevention of cross-contamination.

Table 2

Presence of *Listeria* strains in raw meats and raw and cooked meat products

Samples	Number of samples	Listeria positive samples	*L. m.* positive samples	Reference
Raw sausage	27	26	40	Ralovich and Proksza (1988)
Raw smoked sausage	46	27		
Raw chicken	10		1	Tóth (1988)
Brawn*	3		0	Czirner (1992–1993)
Sausages	13		0	
Frozen meat	2		0	
Salami	1022	840	420	Domján-Kovács (1992–1995)
Dried sausage	204	116	45	
Fermented sausage	12	8	2	
Raw minced meat	3	3	1	
Raw poultry	8	8	7	
Raw poultry liver	106	67	51	
Cubed cooked poultry	67	19	12	
Cooked meat products in casing	17	0	0	
Semi-preserved meat products	203	6	1	
Brawn	55		13	Kiss et al. (1993)
Heat treated sausage	8		2	
Cooked ham	3		1	
Flamed** smoked sausage	8		2	
Minced meat	15		7	

*pork product made of pigs' offal in pig's stomach (similar to Scottish haggis); **flash-heated

Table 3

Presence of *Listeria* strains in raw and pasteurised milk and dairy products

Samples	Number of samples	Number of positive samples: Listeria	Number of positive samples: *L. m.*	Reference
Raw tank milk	25	1	0	Farkas et al. (1988)
Pasteurised milk	50	0	0	
Raw milk	80		3	Rodler and Körbler (1989)
Moulded cheese	10		2	
Other cheese	90		0	
Raw milk	629		81	Beke (1990)
Pasteurised milk and cacao milk	357		2	
Raw tank milk	207	17	12	Ibrahim et al. (1992)
Pasteurised milk	100	0	0	
Chocolate milk	60	7	7	
Soft cheese	15	4	0	
Hard cheese	22	5	1	
Other cheese	30	0	0	
Cream	15	0	0	
Butter	15	2	1	
Raw milk	15		1	Czirner (1992–1993)
Cheeses	18		2	
Human milk	5		0	
Cream	2		0	
Raw milk	4		0	Kiss et al. (1993)
Cream	6		1	
Various cheeses	35		0	
Curd cheeses	21		0	
Butter	1		0	

Table 4

Presence of *Listeria* strains in samples of cold dishes, salads and frozen foods in Hungary

Samples	Number of			Reference
	samples	Listeria	*L. m.*	
		positive samples		
Salads	31		0	Kiss et al. (1993)
Mayonnaise	5		0	
Egg salad	6		1	
Cheese cake	2		1	
Various creams	4		1	
Sandwiches	2		1	
Garnish	5		0	
Deep frozen vegetables	46	38	17	Domján-Kovács (1992–1995)
Deep frozen fruits	14	0	0	
Frozen prefried sliced onion	14	12	4	

Table 5

Presence of *Listeria* strains in ice creams and cream confectionery

Samples	Number of			Reference
	samples	Listeria	*L. m.*	
		positive samples		
Creamy cakes	31		7	Kiss et al. (1993)
Ice creams	15		1	
Sweet creams	9		0	
Chestnut purée	2		0	
Ice creams	324	7	7	Szemenyei (1994)

Table 6

Presence of *Listeria* in the environment of food industrial plants

Samples	Number of			Reference
	samples	Listeria	*L. m.*	
		positive samples		
Dairy plant (P)	20	6	3	Farkas et al. (1988)
Poultry plant	18		3	Tóth (1988)
Dairy farms	49		3	Beke (1990)
Meat factory	191	53		Gasparik-Reichardt et al. (1992)
Dairy plant (B)	50	5	2	Ibrahim (1992)
Ice cream factory	27	8	5	Domján-Kovács (1992–1995)
Five confectionery premises	28	2	2	Szemenyei (1994)

Animal listeriosis. The first animal listeriosis case was verified by Mócsy and Sályi in 1952 in a sheep flock. Since that time *L. m.* has been isolated from dead geese (Csontos et al., 1955), pigs (Csontos et al., 1956), cattle (Csontos and Szemerédi, 1958), rabbits (Vetési and Kemenes, 1965), goats and Cameroon goats (Vetési et al., 1970), monkeys (Vetési et al., 1973), chinchillas (Molnár and Nagy, 1974), roe-deer (Kemenes et al., 1982), nutrias (Hajtós and Malik, 1983), hens (Ivanics et al., 1986), pheasants (Hajtós and Ralovich, 1994), and dogs (Hajtós and Ralovich, 1994). Animal listeriosis is not a reportable and notifiable disease in Hungary; therefore, the real number of these infections is not known. It seems that *L. m.* causes infection most frequently in pregnant sheep. Sheep listeriosis is economically important. Besides sheep listeriosis a few listeric infections in cows have also been diagnosed. Other cases are very sporadic or even unique.

Listeria excretors among healthy animals (pigs, cattle, sheep) have also been observed (Durst and Berencsi, 1975; Forray and Angyal, 1978; Ralovich, 1984). The rate of faecal excretion was 14% among sheep, 27.6% among cattle (Ralovich, 1984), and 25.6% of 144 pigs were excretors (Forray and Angyal, 1978).

Breeding of Listeria-free animals and observance of the rules of good hygienic practice may facilitate the decrease of Listeria contamination of raw materials of animal origin. Excreta and/or secreta of excretors may contaminate the environment including slaughterhouses, dairies, and dairy farms. In a contaminated working place meat, meat products, milk, and dairy products may also be contaminated.

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MYCOPLASMAL PNEUMONIA IN PIGS IN CROATIA: FIRST EVALUATION OF A VACCINE IN FATTENING PIGS

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The immunoprophylaxis of mycoplasmal pneumonia of swine (MPS) caused by *Mycoplasma hyopneumoniae* was investigated for the first time in fattening pigs in Croatia. The incidence of MPS was monitored in pigs weighing on average 27.5 kg (12 weeks old) after immunization with a *M. hyopneumoniae* vaccine. Of 350 pigs in each group, in the nonvaccinated group 55 animals (15.7%) were affected by pneumonia and 11 (3.1%) died of consequences of pneumonia, whereas in the vaccinated group 20 pigs (5.7%) were affected by pneumonia without any death due to the infection. In the nonvaccinated group 44% more pigs were individually treated with antibiotic, and these animals received in-feed therapy for more than 1/4 of the fattening period. Vaccinated pigs gained weight faster, at the rate of 0.745 kg/day (or 82 g/day more) than control animals. The mean score of lung lesions due to *M. hyopneumoniae* was 10.51 in the control pigs and only 0.54 in the vaccinated animals. The total tissue alterations on lungs due to *M. hyopneumoniae, Pasteurella multocida* and/or *Actinobacillus pleuropneumoniae* expressed as the mean score were 13.21 in the control group and 2.98 in the vaccinated group. According to the results of evaluation of the *M. hyopneumoniae* vaccine in the field, the vaccine appeared to provide an adequate immunity in fattening pigs but was less effective when administered to younger pigs at 1–3 weeks of age.

Key words: Mycoplasmal pneumonia, immunoprophylaxis, fattening pigs

Mycoplasmal pneumonia (also termed enzootic pneumonia) of swine (MPS), caused by *Mycoplasma hyopneumoniae,* is one of the most common and economically important diseases occurring in pigs. The disease has been reported from most swine producing areas in the world. In Croatia, MPS was diagnosed by clinical observations, findings of characteristic lung lesions at slaughter, and/or detection of specific antibodies in fattening pigs and gilts in all major farms. Earlier studies of pneumonia in swine herds comprising 1,000 to 6,000 sows revealed

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that 20 to 80% of the gilts were affected with an average of 46.7% serologically positive animals (Bilić and Mihaljević, 1992). A slaughter survey demonstrated lung lesions typical of MPS in 13 to 90% of fattening pigs depending on the prophylactic measures applied (Bilić et al., 1992). These reports are consistent with previous studies in the USA indicating that 30–80% of swine had lesions of MPS (Switzer and Ross, 1975) and that almost all herds (99%) were affected with pneumonia (Muller and Abbott, 1986). The recent studies of pneumonia in Croatia (Bilić and Mihaljević, 1992; Bilić, 1993) have shown that the disease occurring in the field always involves mixed infections, including other agents such as *Actinobacillus pleuropneumoniae* and *Pasteurella multocida,* and that the herds were severely affected according to the classification suggested by Goodwin (1982). MPS is undoubtedly highly influenced by other contributing factors such as poor environment, high stocking density, and use of continuous through-put production systems (Ross, 1990).

There is a direct correlation between severity of clinical picture, mortality and number of culled animals, as well as increased values of feed conversion efficiency of 3.4–4.0 for infected animals in comparison with 2.4–2.6 in *M. hyopneumoniae*-free swine (Stipkovits, 1995). At the same time, the depression in feed efficiency decreases the growth rate from 15 to 12%, and prolongs the fattening period by about 1 month (Pointon et al., 1985).

Apparently, the significant economic losses associated with MPS are primarily due to its chronic effects on grower/finisher performance.

Effective control of MPS has been accomplished since 1991 by vaccination (Bilić et al., 1992) in all large swine farms in Croatia. However, more than 50% of fattening pigs are still produced in small individual units (repopulated without using an appropriate control strategy) that are not covered by such preventive programs (Central Bureau of Statistics, Republic of Croatia, 1994). Therefore, one of the purposes of our field study was to extend the vaccination programme against MPS to units not covered by health monitoring/and MPS disease control programmes.

Protective immunity develops following recovery from the disease or after the administration of adjuvanted vaccines (Ross et al., 1984). Earlier evaluations of *M. hyopneumoniae* vaccines in the field appeared not to provide adequate protective immunity (Kristensen et al., 1981), and in some instances they even appeared to enhance the development of lesions (Ross et al., 1984). The experimental vaccines aiming the control of this important disease were applied in pigs younger than 8 weeks rather than in older animals (Dayalu, 1990; Scheidt et al., 1992; Murphy et al., 1993).

The objectives of this study were (1) to evaluate the effectiveness of a *M. hyopneumoniae* vaccine in 12- to 14-week-old prefattening pigs to provide

protection against MPS during the fattening period, (2) to assess its efficacy in the reduction of gross lung MPS lesions and total lesions, and (3) to record the improvement in weight gain in fatteners.

Materials and methods

Pigs

One privately owned swine fattening unit (Hypor breed) of 700 pigs (350 animals in prefattening phase and 350 animals in fattening phase) located in northwestern Croatia was selected for this study. Twenty-five pigs per group were housed in 14 rearing pens and were given *ad libitum* a grower diet during the 50 days of the prefattening period. In the same unit, 10 animals per group were housed in 35 rearing pens and were allowed to consume their daily portion of liquid feed for the 50 days of the fattening period. All pigs originated from one breeding herd affected with *A. pleuropneumoniae* ser. 2 (frequently observed in Croatia), which herd has never been vaccinated against MPS.

Study design

The study was carried out between October 1993 and April 1994. At the beginning of fattening, the pigs (weighing on average 26.5 kg) were immunized intramuscularly (i.m.) with 2 ml of a *M. hyopneumoniae* vaccine (Respisure®, SmithKline Beecham Animal Health) at 12 and 14 weeks of age. Experimentally vaccinated prefattening pigs (and later fatteners) were mingled with nonvaccinated control animals. The incidence of MPS was monitored daily during the postvaccination period. Medication procedures including individual or mass therapy with antibiotic were performed.

Gross pathology

Gross lesions in lungs were examined at slaughter (day 105 of fattening) in 40 vaccinated and 100 control pigs. A scoring system was applied for the evaluation of tissue damage caused by *M. hyopneumoniae* as described earlier (Goodwin and Whittlestone, 1973). Briefly, the lung lesions due to *M. hyopneumoniae* were scored depending on the intensity of changes on each lung lobe as follows: apical and cardiac lobes were scored from 0 to 10, diaphragmatic and intermediate from 0 to 5. A slightly modified scoring system was applied for the evaluation of tissue damages due to *A. pleuropneumoniae* and/or *P. multocida*. A single haemorrhagic abscess caused by *A. pleuropneumoniae* and each localized adhesion caused by *P. multocida* between apical, cardiac, and diaphragmatic lobes, were scored as 1.

The adhesions observed on the intermediate lobes were scored as 0.5, localized adhesions on one of the lobes were scored as 1, and diffuse adhesions covering the whole lobe were scored as 2.

Results

Twenty of 350 vaccinated pigs developed clinical signs of MPS. The vaccinated pigs gained weight at the rate of 0.745 kg/day, and they were on average 8.0 kg heavier at day 105 of fattening than nonvaccinated control pigs (Table 1).

Table 1

Experimental design and summary of production data in fattening pigs vaccinated against MPS*

Variable	Vaccinated** group	Control group
No. of pigs	350	350
Average weight at day 0 (kg)	27 (+/– 2.5)	28 (+/– 2.7)
No. of pigs with clinical signs of pneumonia	20 (5.71%)	55 (15.71%)
No. of pigs culled due to pneumonia	0	11 (3.14%)
No. of individual treatments with antibiotic	24 (6.86%)	178 (50.86%)
No. of days with in-feed therapy	0	26
Average body weight at day 105 of fattening (kg)	105.7	97.7
Average daily weight gain per pig (kg)	0.745	0.663

* The duration of the fattening period was 105 days on the average.
** 2 ml of SmithKline Beecham Animal Health vaccine was given i.m. at 12 (day 0) and 14 weeks of age

Fifty-five of the controls (15.7%) developed MPS, and 11 of them (3.14%) were culled due to the consequences of the disease. In the control group, 44% more pigs were individually treated with antibiotic and underwent additional in-feed therapy for 26 days. Fifty-one % more vaccinated pigs were free of lung lesions, and 40.4% more of their lung lobes examined were without visible tissue damages that could be ascribed to *M. hyopneumoniae* (Table 2). In the control pigs, gross pathologic changes caused by *M. hyopneumoniae* typically affected 3, 6 or 7 lobes, while in the vaccinated animals characteristic lesions were typically observed in 1 or 2 lobes (Table 2). The average score of lung changes caused by *M. hyopneumoniae* was 0.5 in the vaccinated pigs and 10.5 in the control animals.

The total score of changes due to all infective agents studied (*M. hyopneumoniae*, *A. pleuropneumoniae* and/or *P. multocida*) was 3.0 in the vaccinated group and 13.2 in the control group of pigs.

Table 2

Gross pathologic observations of lung lesions in fattening pigs on the slaughtering line

Variable	Vaccinated group	Control group
No. of pigs examined	40	100
% of MPS-positive pigs	25.0	76.0
% of lung lobes with MPS lesions	7.9	48.3
Number of lung lobes infected		
0	75.0	24.0
1	10.0	6.0
2	7.5	8.0
3	2.5	19.0
4	2.5	6.0
5	2.5	7.0
6	0	10.0
7	0	20.0
Mean score of MPS lesion	0.54	10.51
Mean score of total lesions*	2.98	13.21

* Total lesions caused by infection with *M. hyopneumoniae*, *A. pleuropneumoniae* and/or *P. multocida*

Discussion

Improved control of MPS is possible by the use of a *M. hyopneumoniae* vaccine when applied early in the fattening period (12 weeks of age) under optimum environmental conditions and at proper stocking density. Use of a strict all-in, all-out production scheme is the most effective way to control MPS in infected herds. However, in spite of good environmental conditions including air quality, ventilation and temperature, the pigs in the studied farm were housed in buildings with older infected pigs, and had more pneumonia (due to *M. hyopneumoniae* and/or *A. pleuropneumoniae*) at slaughter than those housed by age groups as demonstrated before (Clark et al., 1989). Those authors recommended no more than 3 weeks variations in age in stocking a building.

There is strong evidence that mixed infections with *M. hyopneumoniae* and *A. pleuropneumoniae* resulted in suppressed phagocytic ability of alveolar macro-

phages (Yagihashi et al., 1984; Caruso and Ross, 1990). This suppression may contribute to the predisposition of MPS diseased pigs to bacterial pneumonia caused by *P. multocida,* a common secondary invader in swine (Ciprian et al., 1988). Lymphocytes from bronchial lymph nodes of pigs infected by *M. hyopneumoniae* had reduced ability to produce antibodies to unrelated antigens (Wannmuehler et al., 1988).

Clinical results in the vaccinated fatteners correlated well with the extent of lung lesions observed at slaughter. It is likely that the course of MPS and the time required for the lesions to be present is probably influenced by the severity of the disease and the involvement of other agents such as *A. pleuropneumoniae* and *P. multocida.* Apparently, the pigs immunized against MPS were also better protected against other agents, especially *A. pleuropneumoniae*. We observed an almost complete resolution of the gross lesions in 75% of the pigs by day 100 after possible exposure to the causal agent(s) of pneumonia.

Although vaccination of older pigs at the beginning of fattening (at 12 to 14 weeks of age) was recommended neither by earlier reports (Lam and Switzer, 1971; Goodwin and Whittlestone, 1973; Murphy et al., 1993) nor by the producer of vaccine (who suggested that the optimal age for vaccination was 1 to 3 weeks), our results indicated that the later vaccination indeed increased the resistance of pigs to MPS but was less effective than in cases when administered to younger pigs (Bilić et al., 1992). Moreover, the vaccination of pigs against MPS enhanced the resistance of immunized animals to other organisms contributing to the severity of lung infection, i.e. *A. pleuropneumoniae* and/or *P. multocida* as detected in our survey. The overall data from this study were checked a few times in the same swine farm and were confirmed by even better production performances. At the same time, the economic impact of antibiotic therapy favours the application of vaccines for the control of MPS if such effective, whole-herd immunization presents the possibility of enhancing pig performance while reducing *M. hyopneumoniae* population densities and thereby reducing losses due to MPS and multifactorial pneumonia in swine. To our knowledge, vaccination of pigs against mycoplasmal pneumonia has not been reported in 12-week-old animals. Thus, it is encouraging that our attempt to protect 3-month-old pigs resulted in substantially reduced clinical signs and lesions caused by virulent *M. hyopneumoniae.*

Acknowledgement

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REMARKS ON THE MORPHOLOGY, SITE OF INFECTION AND VALIDITY OF SOME COCCIDIAN SPECIES FROM FISH

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The morphological identification of carpelli-type oocysts of *Goussia* species parasitizing the gut of different cyprinids is unreliable. Species similar in shape and size can be differentiated only by cross-infection experiments. *Goussia scardinii* (Pellérdy et Molnár, 1968), developing in the renal tubules of cyprinids, is a synonym of *Goussia leucisci* (Shulman et Zaika, 1964). The morphology and location hitherto assumed to be species characteristics of *G. scardinii* can be explained by the aging of oocysts and the type of host reaction. *Eimeria rutili* Dogiel et Bychowsky, 1938 is a coccidian parasite occurring in various organs of the roach. Its oocysts can be found in the blood vessels and, thus, also in the gill capillaries. *Eimeria branchiphila* Dyková, Lom et Grupcheva, 1983, described from blood vessels of the gills of the same fish species, should be regarded as a synonym of *E. rutili*. The *Octosporella* species described from fish are based on erroneous identification and should be considered *nomina nuda*. Similarly, the *Eimeria* and *Isospora* species described from fish by Davronov in 1987 should also be regarded as *nomina nuda*, as they appear to be avian or mammalian coccidia that entered the intestinal tract of fish with food. The sporocyst residua of fish coccidia cannot be used as characters suitable for species identification, as they tend to change with oocyst aging.

Key words: Fish coccidia, *Eimeria, Goussia,* synonyms and development of fish coccidia, host reaction, site of infection

True coccidia living in fish are relatively little studied parasites, despite the fact that by now the number of known species of the genera *Eimeria* Schneider, 1875, *Goussia* Labbé, 1896, *Cristallospora* Labbé, 1896, *Calyptospora* Overstreet, Hawkins et Fournie, 1984 and *Cryptosporidium* Tyzzer, 1907 has exceeded 200. These parasites have a variety of properties different from those reported from mammals and birds, and the differences can be attributed both to the aquatic environment and to the hosts' changing body temperature (Lom and Dyková, 1992; Davies and Ball, 1993; Molnár, 1995). Despite the high variability characterising their development, coccidia show a high degree of uniformity regarding oocyst structure. This is why attempts to classify coccidian species of

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mammals and birds (e.g. Pellérdy, 1964) have failed, and the majority of the several thousand known species are still assigned to the genus *Eimeria*. Fish coccidia, on the other hand, exhibit relatively well-detectable differences in oocyst structure, which have been used for assigning them to the genera *Eimeria, Goussia, Calyptospora, Cristallospora* and *Cryptosporidium.* Attempts have been made at a further categorization of coccidia based upon the intracellular location of the given species. On these grounds, Dyková and Lom (1981) proposed *Epieimeria* while Daoudi (1987) tried to establish *Nucleoeimeria* and *Nucleogoussia* as separate genera.

Based upon studies encompassing many years, the present paper discusses some problems and erroneous statements regarding the taxonomy, development and host specificity of fish coccidia reported as a result of ill-substantiated observations.

Materials and methods

Research into fish coccidia commenced in Hungary about 30 years ago. Besides species known from the literature, new coccidia were also described (Pellérdy and Molnár, 1968, 1971). The methodology enabling successful collection of fish coccidia was described by Molnár (1977). Based on the works of Molnár (1982, 1989) it is known that although the majority of fish coccidia sporulate endogenously, there are numerous coccidia which leave the fish unsporulated. These latter species can be induced to sporulate in tap-water and, thus, their taxonomic classification becomes possible.

The present paper is based primarily upon recent surveys of the parasite fauna of fish species living in Lake Balaton; however, in some cases, when justified by problems to be settled, I have also re-examined materials fixed earlier (Pellérdy and Molnár, 1968; Molnár and Baska, 1986; Molnár, 1989).

In the majority of cases, coccidial infection was diagnosed by microscopic examination of mucus and mucosal scrapings collected from the gut and of squash preparations made from different organs. In positive cases, the organs were fixed in 10% formalin or Bouin's solution for histology, embedded in paraffin, cut into 4–6 μm thick sections, and the sections stained with haematoxylin and eosin or by Farkas-Mallory technique. Earlier, the morphological characteristics of oocysts collected in sporulated state, or caused to sporulate in tap-water were studied under a Zeiss Jenaval microscope and recorded with the help of a drawing tube. More recently, drawings are made of oocysts recorded on videotape under an Olympus microscope, projected to a screen and magnified, and their measure-

ments are related to the scale of an objective micrometer projected to the screen in the same way. The microphotographs were taken using a Zeiss-Jenaval photomicroscope.

Results and discussion

1. Coccidia causing carpelli*-type coccidiosis*

Goussia species causing so-called dispersed coccidiosis and characterized by similar oocyst size but a somewhat different sporocyst structure are frequently detected from the gut of European cyprinids. Oocysts of such species, which occurred in dispersed locations, measured 7–13 μm in diameter and sporulated in the host's gut, were found in the gut of almost all cyprinids in most of the present investigations carried out in different periods of the year. Of these species, *Goussia carpelli* (Leger et Stankovitch, 1921) parasitizing the gut of the common carp and crucian carp was well distinguishable from *G. sinensis* (Chen, 1956) living in the gut of the silver carp and bighead; however, in the case of *Goussia* species parasitizing the gut of *Abramis, Blicca, Gobio, Phoxinus, Rutilus, Barbus* species and hardly differing from *G. carpelli* it was practically impossible to make a reliable species identification based upon morphological examinations. Oocysts found in these fish bore morphological characters typical of *G. cyprinorum* (Stankovitch, 1921), *G. legeri* (Stankovitch, 1920), *G. stankovitchi* (Pinto, 1928) and *G. cylindrospora* (Stankovitch, 1921) which are species close to *G. carpelli*. The intraspecific morphological variability and the similarity of oocysts found in different fish species render it difficult to establish a reliable diagnosis. Therefore, as Lukeš et al. (1991) pointed out, certain authors identified the oocysts found in different fish species with *G. carpelli*. According to our observations, differences between oocysts found in different cyprinids are difficult to detect but undoubtedly exist. For instance, *G. cylindrospora* is easily distinguishable from *G. carpelli* by the looser structure of its oocyst, while from *G. legeri, G. stankovitchi* and *G. cyprinorum* it differs only in the more elongated, slightly cylindrical shape of its sporocyst. This is why many authors regard these latter species as synonyms. In the case of these species having a small oocyst, host specificity cannot be reliably determined by morphological examinations. Only cross-infection experiments could furnish evidence as to whether we are dealing with species closely resembling one another or with variants of a single species. Up to now, reliable experiments of this kind have been carried out only by Lukeš et al. (1991), who demonstrated that *G. carpelli* coccidiosis of common carp could be transmitted only to the closely related fish species *Carassius auratus* experimentally. Coccidia of "*carpelli*" type seem to have a somewhat narrower host specificity, which means that a given parasite can infect only the taxonomically most closely related hosts. Luckily, in

the case of coccidia developing according to this short cycle infection experiments can be carried out successfully. Namely, Paterson and Desser (1982) found that infection could be established successfully in a direct manner in suitable hosts. According to Molnár (1979), Kent and Hedrick (1985), and Steinhagen and Körting (1990), the use of tubificid vectors can intensify an infection.

2. The Epieimeria *problem*

This genus was created by Dyková and Lom (1981) for those coccidia which develop intracellularly but extracytoplasmally under the cell membrane of the host's enterocytes (Fig. 1). In addition to the above type of merogony and gamogony, Lom and Dyková (1992) regard also intracellular sporogony as a typical character of the genus. On the contrary, Molnár and Baska (1986) demonstrated that in the majority of cases sporogony took place in extracellular location. Benajiba et al. (1994), who synonymized *Epieimeria* with *Eimeria*, concluded that in heavy infections of the eel with *Eimeria anguillae* Leger et Hollande, 1922 mostly extracellular sporogony takes place, while in less severe infection the oocysts get into the deeper layers and finish sporulation beneath the epithelial cells. Molnár (1984) and Benajiba et al. (1994) explained the "sinking" of oocysts to subepithelial site by the effect of host reaction. In this case the uninfected neighbouring host cells overgrow the parasitized cells and press them down to the deeper layers (Fig. 2).

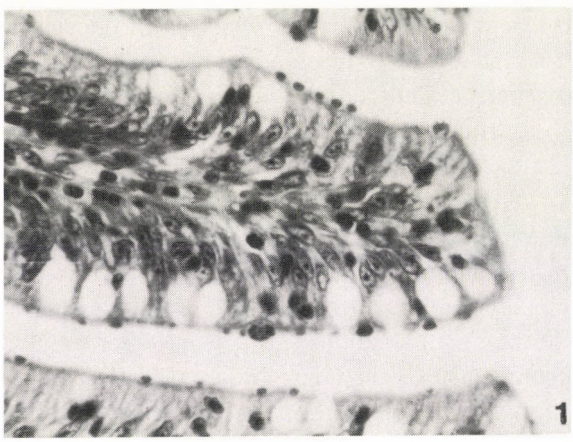


Fig. 1. Epiplasmally developing young trophozoites of *Eimeria anguillae* in the gut epithelium of an eel. × 500

When studying *E. anguillae* infection of the eel, I found that in the majority of cases epicellular merogony and gamogony were followed by the direct release

of unsporulated oocysts into the gut lumen and their sporulation either there or outside the host. Less frequently, intercellularly sporulated oocysts were seen beneath the epithelial layer after epicellular merogony and gamogony. This observation is consistent with the findings of Benajiba et al. (1994). At the same time, no correlation could be demonstrated between the presence of the latter oocysts and the intensity of infection. On the contrary, in some cases when highly intensive *E. anguillae* infection was detected in histological preparations, epicellular meronts and trophozoites (Fig. 3) were seen next to intracytoplasmally located macrogamonts and unsporulated oocysts (Fig. 4).

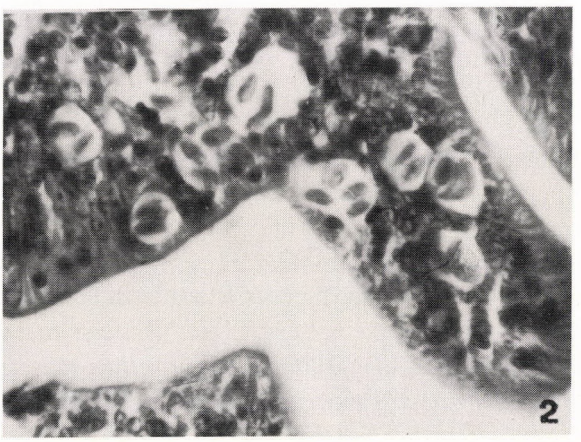


Fig. 2. Subepithelially located sporulated oocysts of *E. anguillae*. × 1000

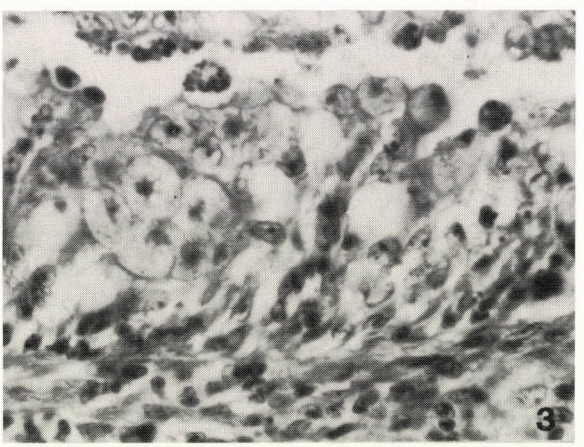


Fig. 3. Hyperintensive infection with *E. anguillae* in the gut of an eel. Besides epiplasmally located developmental stages, some macrogamonts have got into intercellular position in the regenerating epithelium. × 1000

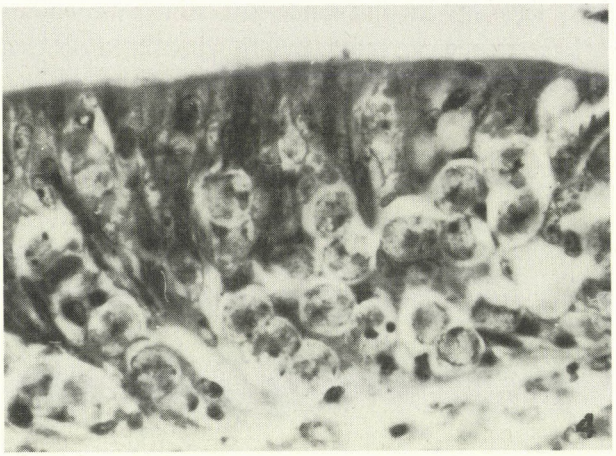


Fig. 4. Inter- and subepithelially located unsporulated oocysts of *E. anguillae* in the gut epithelium of an eel. × 1000

A similar case was reported by Kent et al. (1988) who described *Goussia girellae* from *Girella girella*. These authors found both epicellularly and intracytoplasmally located gamonts in the gut epithelium. It seems that epicellular development, which is typical of *E. anguillae* and *G. girellae*, may change into an intracytoplasmal type during late gamogony as a result of the host reaction.

3. Goussia *spp. infecting renal tubules*

Of eimeriids living in the kidney, the first species to become known were *Goussia hypophthalmichthys* Dogiel et Achmerow, 1959, *G. amurensis* Dogiel et Achmerow, 1959, *G. strelkovi* Shulman et Zaika, 1962 and *G. leucisci* Shulman et Zaika, 1964 reported from the Far East and Siberia. Subsequent additions to the group included *G. scardinii* described by Pellérdy and Molnár (1968), *G. freemani* reported by Molnár and Fernando (1974), and *G. spraguei* described by Morrison and Poynton (1989). In Hungary, oocysts morphologically identified as *G. leucisci* or *G. scardinii* are very often found in the epithelium of the renal tubules of cyprinids in March and April. The prevalence of such oocysts in bream, white bream, roach and rudd living in Lake Balaton was found to be 35%, 42%, 33% and 80%, respectively. The kidney parasite described by Lukeš (1993) from golden carp, which morphologically also corresponds to *Goussia leucisci*, had a prevalence of 100% in gibel carp (*Carassius auratus gibelio*) in Little Balaton (Kis-Balaton). In fish species of the river Danube, oocysts of *G. leucisci* and *G. scardinii* type (Figs 5, 6 and 7) were detected in the rudd, nase, and orfe. Regarding these parasites, the following two fundamental questions arise: (1) Do the

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morphologically very similar *Goussia* oocysts developing in the renal tubules of different fish species belong to a single or to several species? (2) Can *Goussia scardinii* (Pellérdy and Molnár, 1968) be considered a valid species? Several arguments can be advanced for both an affirmative and a negative answer to these questions.

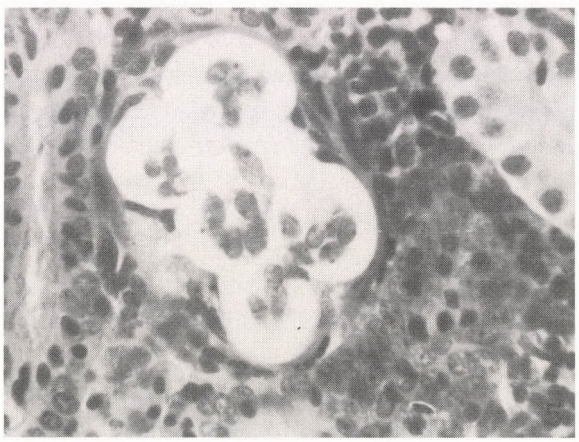


Fig. 5. Oocysts of *Goussia.leucisci* with "*scardinii*"-type rounded sporozoites in the obliterated tubuli of a rudd. The epithelial cells of the renal rubules are markedly flattened. × 1000

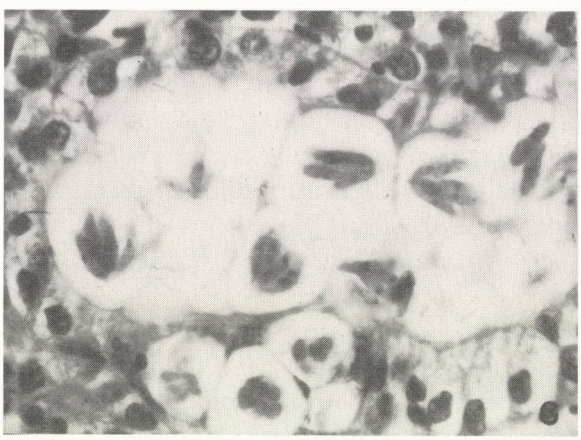


Fig. 6. Goussia leucisci-type oocysts with banana-shaped sporozoites in the renal tubules of a gibel carp. × 1000

It is indisputable that *G. muraiae* Molnár, 1978 and *G. spraguei*, which develop in the same location as *G. leucisci,* resemble it morphologically but differ

from it in some characters, and infect taxonomically distant hosts, are distinct species. At the same time, it is questionable whether the taxonomically more distant gibel carp is a suitable host for *G. leucisci*, a species originally described from leuciscids. According to Jastrzebski (1984) and Lom et al. (1989), *G. leucisci* is a parasite of broad host spectrum: in addition to Eurasian species, it parasitizes North American cyprinids as well. Thus, *G. freemani* (Molnár and Fernando, 1974) should also be considered one of the synonyms of the above coccidium. The validity of *G. scardinii* has not been queried as yet. According to the original description (Pellérdy and Molnár, 1968), this species differs from *G. leucisci* both in morphology and location. Oocysts of *G. scardinii* contain short ellipsoidal, stubby sporozoites (Fig. 5) at the poles of the sporocyst, while oocysts of *G. leucisci* have banana-shaped sporozoites (Fig. 6) in head-tail arrangement. The researchers originally describing *G. scardinii* gave the renal parenchyma as its location; *G. leucisci*, on the other hand, is a typical parasite of the tubular epithelium. Studies on the process of *Goussia* infection of the kidney demonstrate, however, that the seemingly important differences between the two species are in fact virtual. The shape of sporozoites is a trait typical of a certain developmental stage, rather than being a species character. When monitoring the oocyst sporulation process by microscopy, the present author often observed that banana-shaped sporozoites suddenly shrank and irreversibly assumed the shape typical of the oocysts of *G. scardinii*. Similarly, in our cases the sporozoites of oocysts found in the renal tubules of gibel carp showed a typical "*leucisci*" shape, which is at variance with the typically shrunken shape of sporulated oocysts as seen on the photographs taken by Lukeš (1993). The re-examination of histological preparations, including those that served for the original description, shows that the location of *G. scardinii* oocysts in the renal parenchyma is the result of a secondary process in which the obstructed and damaged tubular segment containing oocysts becomes surrounded by proliferative tissue consisting of macrophages and connective tissue (Fig. 7). Thorough examination of such tissue formations, which occasionally contain also unsporulated oocysts, consistently reveals a flattened tubular epithelium.

Due to the above facts, the species *G. scardinii* described from *Scardinius* and *Blicca* should be regarded as a synonym of *G. leucisci*. It could be re-created as a distinct species only if further investigations prove that *G. leucisci* was a collective species, and that the kidney of cyprinids was parasitized by numerous *Goussia* organisms of similar structure but belonging to different species. This possibility is suggested first of all by the species *G. polylepidis* described by Alvarez-Pellitero and Gonzalez-Lanza (1985), which differs from "*leucisci*" type oocysts also structurally.

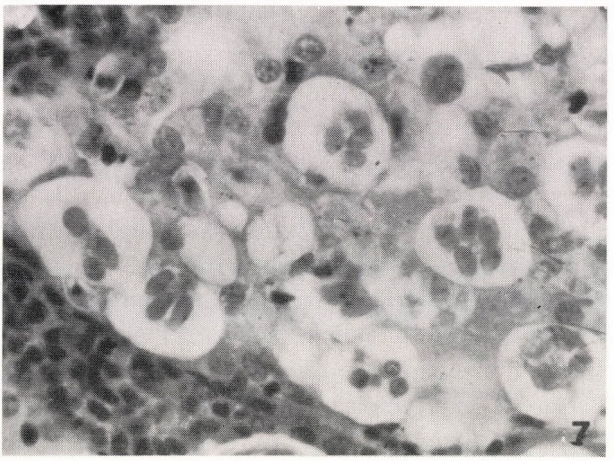


Fig. 7. Advanced stage of coccidian infection in the kidney of a rudd. The "*scardinii*"-type oocysts of *G. leucisci* are infiltrated by light-staining epithelioid macrophages. × 1000

4. The Eimeria rutili *problem*

Eimeria rutili Dogiel et Bychowsky, 1938 is a parasite easily identifiable by its characteristic capped sporocysts. It is often demonstrated in the kidney of *Rutilus rutilus* in Hungary. This parasite is widespread in areas ranging from the Ural Mountains to Spain (Shulman, 1984; Alvarez-Pellitero and Gonzalez-Lanza, 1986). As, due to its flexible wall, the shape of the parasite's oocyst may vary from round to elongated ellipsoidal, only the sporocysts are suitable for species identification. Although the species *E. esoci* Shulman and Zaika, 1962 resembles *E. rutili* in sporocyst size and shape, its occurrence in a taxonomically very distant fish species (*Esox lucius*) probably justifies its description as a valid species. The fact that this parasite is located in the wall of the gut and urinary bladder rules out the possibility of its oocysts having entered that predatory fish passively, via some food fish species. In addition to the above two species, parasites with similar capped sporocysts have been reported in Europe by Molnár (1978) from the internal organs of *Alburnus alburnus,* by Dyková et al. (1983) from *Rutilus rutilus*, and by Alvarez-Pellitero and Gonzalez-Lanza (1986) from *Carassius carassius,* and have been described by the above authors as *E. nemethi* Molnár, 1978, *E. branchiphila* Dyková, Lom et Grupcheva, 1983 and *E. baueri* Alvarez-Pellitero et Gonzalez-Lanza, 1986 respectively. While, however, *E. nemethi* and *E. baueri* found in different hosts differed from the typical *E. rutili* also in sporocyst dimensions, *E. branchiphila* did not, and its description as a distinct species was justified only by its occurrence in the gills.

Our studies on the occurrence and dynamics of fish coccidia living in the kidney have revealed that oocyst formation of *E. rutili* always took place in the renal parenchyma and never in the tubules (Fig. 8). Similar observations were made by Alvarez-Pellitero and Gonzalez-Lanza (1986). The close connection of oocyst formation with the blood path can be demonstrated histologically. This explains why oocysts can be found in the liver, spleen and gills, in addition to the kidney, in the period of sporulation. From the available data it cannot be definitely determined whether the oocysts of *E. rutili* are formed exclusively in the kidney and then carried to the liver, spleen and gills by the blood stream, or they are formed in all organs abundantly supplied with capillaries. Based upon the morphological identity and the unique development of *E. rutili* it seems to be unquestionable that *E. branchiphila* described by Dyková et al. (1983) is synonymous with the species *E. rutili*, and that the oocysts found in the gills represent the stages of this latter species showing a unique way of excretion from the host. For that matter, the development of *E. branchiphila* in the endothelium was suggested already by Dyková et al. (1983) who, by the analogy of *E. brevortiana* Hardcastle, 1994, considered it probable that developmental stages were translocated from some other organ via the blood stream.

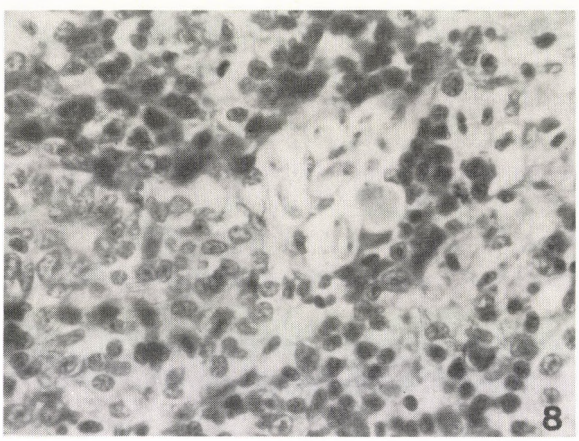


Fig. 8. Sporulated oocysts of *Eimeria rutili* in the renal parenchyma of a roach. × 1000

5. The Octosporella *problem*

The genus *Octosporella* was created by Ray and Raghavaghari (1942) for a coccidium occurring in a lizard. From fish, Li and Desser (1985) described three species belonging to this genus. The validity of the genus *Octosporella* Ray et Raghavaghari, 1942, at least as regards its occurrence in fish, is highly question-

able because of the inaccuracy of the existing descriptions. The stages seen in the photographs published by Li and Desser (1985) are actually mature *Goussia* oocysts in which the sporocysts have opened along their sutures, and each of the formations marked as sporocysts corresponds to a sporocyst valve. The present author successfully produced similar formations by exerting mechanical pressure on aged oocysts of *G. degiustii* Molnár et Fernando, 1974 and *G. freemani* derived from *Notropis cornutus*. In view of the above considerations, in the author's opinion *Octosporella notropis* Li et Desser, 1985, *O. opeongoensis* Li et Desser, 1985, and *O. sasajewuensis* Li and Desser, 1985 should be regarded as *nomina nuda*.

6. Fish coccidia extraneous to the species from which they are isolated

The intestinal content of fish, first of all that of predatory fish species, frequently contains oocysts derived from other animals ingested as food. After the excretion of faeces the chance of finding such extraneous coccidia in the mucus collected from the gut epithelium is minimal. Davronov (1987), who studied the coccidiofauna of fish in Uzbekhistan, isolated numerous thick-walled oocysts from the faeces of fish by a flotation technique. He described six *Eimeria* species under the name of *E. amudarinica, E. syrdarinica, E. varicorhini, E. barbi, E. schizothoraci,* and *E. siluri*. In addition, he reported the occurrence of three *Isospora* spp. The collection method applied and the different structure of the oocysts as compared to that of the known fish coccidia unquestionably indicate that the above-cited author must have isolated coccidium species of other animals, probably aquatic birds, from the faeces of fish. Therefore, the species described by him should be regarded as *nomina nuda*.

7. The problem of the sporocyst residuum

The oocyst and sporocyst residuum, its shape, size and compact, finely or coarsely granular structure are important taxonomical characters which remain constant within the sporulated oocyst over a very long period. These traits are often used as taxonomic characters also for fish coccidia. At the same time, it is known that in the case of fish coccidia this residual material undergoes very rapid shrinking, and at room temperature it disappears from the oocyst in a few days' time. The sporocyst residuum cannot be used as a taxonomic character but is very useful in judging the age of oocysts and the stage of their sporulation.

Acknowledgements

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INFLUENCE OF A DEFICIENT INTAKE OF HIGH AND LOW DEGRADABLE PROTEIN ON BODY COMPOSITION, METABOLIC ADAPTATION, PRODUCTION AND REPRODUCTIVE PERFORMANCE IN EARLY LACTATION DAIRY COWS

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High-yielding dairy cows (n = 34) were divided into high (HD) and low (LD) protein degradability groups after delivery, and used for a feeding trial which lasted up to day 80 ± 10 of lactation. The cows were regularly weighed, their body condition was scored (BCS), and their reproductive status assessed. Blood samples were taken simultaneously, and ruminal fluid was collected on the last day of the trial. On postpartum day 45 ± 6 and 11 days thereafter two prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) injections were administered. The ammonia and urea concentrations of the ruminal fluid were higher in cows of group HD. The C2:C3 ratio was 2.7:1 vs. 2.3:1 in HD and LD cows, respectively. The amino acid and lipid composition of blood reflected the dietary treatments. The BCS and blood urea concentration were slightly higher in HD animals. The average daily milk production of LD cows was 1.5 kg higher than that of HD cows. The pregnancy rates of the two groups at day 200 after calving did not differ, but LD cows showed their first visible oestrus and reconceived significantly ($p < 0.05$) later. It can be concluded that — at a marginal energy supply — a moderate (13%) deficiency of rumen-degradable protein during the first 10 weeks after calving can be more detrimental to reproductive performance than a severe (27%) deficiency of undegradable protein.

Key words: Protein degradability, blood and ruminal fluid parameters, body condition, body composition, urea space, progesterone profile, conception rate, postpartum period

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Recent developments in the feeding strategy of high-yielding dairy cows to express protein requirements have placed an emphasis not only on the crude protein content of the diet, but also on its ruminal degradability. Scientists indicated a separate N requirement for rumen bacteria and for amino acids reaching the small intestine (NRC, 1985). In the last two decades, average milk production has increased by 300% in Hungary, reaching a level of 7000–9000 kg/year in the good herds. Studies relevant to this field have dealt with the energy supply of the dry cow (Pethes et al., 1985), the effect of varying energy to protein ratios (Magdus et al., 1992), the degradability of protein (Veresegyházy et al., 1989, 1993), and the interaction between nutrition and reproduction (Huszenicza et al., 1988*a*, 1988*b*, 1988*c*).

The body condition scoring (BCS) system is a subjective but reliable method for accurately determining the amount of energy stored in the body reserves of live dairy cows. Body condition pertains to the degree of body fat. There is a need for concern with each stage of the lactation cycle (Otto et al., 1991). High-producing dairy cows in early lactation utilise their tissue reserves to support milk synthesis. BCS can serve as a useful tool for assessing the energy balance.

Dilution techniques have proven to be useful for the *in vivo* determination of body composition (NRC, 1967; Forbes, 1987). For example, urea is distributed via the vascular system and diffuses passively throughout the whole body water. Therefore, the urea space reflects the total body water. Urea space correlates with the water space and body fat in cattle 12 min after intravenous injection of a single dose, and was proposed for the estimation of body composition (Painter, 1940; Preston and Kock, 1973). Valuable estimates of body composition have been obtained (Kock and Preston, 1979; Bartle et al., 1987; Hammond et al., 1988; Hammond et al., 1990).

The nutritional status (body composition) of dairy cows is closely related to the calving-to-conception interval and the reconception rate. The feeding of excess crude protein, rather than a deficient protein supply, is known to exert a detrimental effect on reproduction in dairy cattle (Elrod and Butler, 1993; Elrod et al., 1993). The deficiency of protein sources has been demonstrated to be a factor adversely affecting fertility only in suckling beef cows kept under extreme climatic conditions. Insufficient protein supply may also occur, however, in high-yielding dairy cows during the first weeks of lactation.

Data available on the interaction of protein supply and fertility are inconsistent. As Ferguson and Chalupa (1989) have pointed out, the age of the cow, the energy concentration of the ration, the intake of undegradable protein, and the uterine health status influence the effect exerted by varying levels of crude protein supply. They stressed the need for further trials to elucidate the role that an excess or deficient supply of degradable and undegradable crude protein may have on the

conception rate (CR). The aim of this experiment was to study the influence of high and low degradable protein intake on postpartum metabolic adaptation and ovarian function as well as on reproductive performance in high-yielding dairy cows fed a marginally protein-deficient diet.

Materials and methods

Location of the study. Animals

This study was carried out in Hungary, on a well-managed commercial dairy farm where the average milk production of the about 1200 Holstein-Friesian cows had been around 8000 kg per lactation for many years. The animals were kept in a total confinement system in free-stall barns, in groups of 80 to 110, formed on the basis of the stage of reproduction and lactation and actual daily milk production. Neither pasturing and regular physical exercise (with the exception of walking to and returning from the nearby milking house) nor individual feeding was technically possible. There was no difference in the pretrial feeding programme, i.e. in the nutrition provided during the dry period. In the first 6 weeks of the dry period the cows received 12 kg corn silage and 6 kg meadow hay, with 2 kg of wheat bran supplementation in the last two weeks.

All of those second to fifth parity cows which (1) produced at least 8000 kg milk (corrected for 3.5% fat content) during the previous lactation and (2) more than 40 kg FCM per day in the first 12 weeks after their previous delivery, and (3) calved within a 2-week period in late February to early March after an uncomplicated gestation of 275 to 285 days duration were involved in this study (in total: 34 cows). Further prerequisites of the cows' selection for this study were as follow: (4) no need for manual assistance at calving, and (5) an uncomplicated, spontaneous expulsion of fetal membranes within 6 h. The animals were assigned into two groups receiving one of the two diets from the first week after delivery. The groups were formed so as to be identical in parity and daily milk yield at the beginning of the previous lactation.

Nutrition

From the end of the first postpartum week the groups of cows received one of the experimental diets (Table 1) designated high and low degradable protein rations (hereafter: HD and LD, respectively) according to the degradability of their concentrate proteins. (The HD ration corresponded to the ration previously fed at the farm; the LD ration was formulated by changing the ingredients in the concentrate.) To investigate the effect of various amounts of rumen degradable and undegradable protein, diets were designed to provide the following percentages of

degradable and undegradable protein relative to the NRC requirement: diet HD, 100, 87%; diet LD, 73, 110%. All cows were fed a total mixed ration *ad libitum*. The concentrate consisted of various amounts of protein sources (e.g. blood, fish and corn gluten meal) to alter the degradability of protein. The daily ration was distributed six times a day, practically *ad libitum*. The feeding trial lasted till day 80±10 of the lactation. The average daily milk production was predicted to be about 40 kg of milk of 3.5% fat content, so the energy requirement was expected to be met in both groups. However, as regards the protein supply compared to the NRC (1989) recommendations, the cows of group HD received a ration severely deficient (–16% of CP) in undegradable protein (UDP), while those of group LD were fed a diet characterised by a moderate lack (–8.0% of CP) of rumen degradable protein (RDP) sources (Table 2 and Fig. 1). The UDP fraction was increased by giving fish, blood and corn gluten meal, like in the trial of Rusche et al. (1993).

Investigation of metabolic and ruminal response

When the cows were divided into two feeding groups during the first week after calving, they were weighed and their body condition was estimated and scored by two independent evaluators, according to the Virginia Polytechnic system (Wildman et al., 1982), modified by Edmonson et al. (1989). Simultaneously, before the morning feeding, blood samples were taken from the jugular and the coccygeal veins to analyse some haematological parameters (PCV, haemoglobin), as well as some enzymes and metabolites such as urea, creatinine, glycohaemoglobin, fructosamine, total protein, albumin, glucose, non-esterified fatty acids (NEFA), acetoacetate (ACAC), aspartate aminotransferase (AST), cholic acid, total cholesterol (TCh), HDL-cholesterol (HDL-Ch), and triglycerides, reflecting the energy, lipid and protein metabolism as well as the liver and kidney functions. All these examinations were repeated 3, 5 and 8 weeks later (i.e. on day 24 ± 6, 38 ± 6 and 59 ± 6 after calving) and subsequently on day 10 after the 4th sampling. (The last two samplings were scheduled to take place at the expected time of the $PGF_{2\alpha}$-synchronised oestruses and at the subsequent mid-luteal phases.) Some days later, on the last day of the experimental feeding period, ruminal fluid samples were obtained by stomach tube technique before the first morning feeding to analyse the pH as well as the ammonia, urea, lactic acid and VFA levels. At the same time, blood samples were collected to determine the total free amino acid levels, free amino acid profiles and lipid composition. In addition, blood (plasma) samples were collected at 3- to 4-day intervals throughout the experimental period to analyse the actual concentrations of thyroid hormones (T_3, T_4) and cortisol.

Table 1

Ingredients, chemical composition and nutritive value of diets fed to postpartum cows

Ingredients[1]	IFN[2]	Amount, kg/d	
Corn silage	3-20-506	14.0	
Alfalfa hay	1-00-063	6.0	
Wheat bran	4-05-190	0.5	
Corn meal	4-02-861	0.5	
Concentrate (HD or LD)		10.2	
		HD ration[3], %	LD ration[4], %
Barley	4-00-549	70.18	–
Corn	4-02-861	–	73.53
Corn gluten meal	15-02-900	–	4.90
Pea	5-03-600	17.54	–
Soybean meal	5-04-596	8.77	9.80
Blood meal	5-00-380	–	4.90
Fish meal	5-02-000	–	4.90
Mineral-vitamin premix[5]		3.51	1.97
Dry matter, kg		20.10	20.10
NE_l, MJ		142.49	147.67
Crude protein, g		2927	3380
RDP, g		2117	1862
UDP, g		810	1518
Rumen degradability[6] of protein, %		72	55

[1]Composition expressed on an "As Fed Basis"; [2]IFN = International Feed Number; [3]HD = High degradable crude protein; [4]LD = Low degradable crude protein ration, % as fed basis; [5]Contained wheat bran and 200 g mineral and vitamin mix (13% calcium, 6.5% phosphorus, 9.5% sodium, 3.0% magnesium, 650,000 IU/kg vitamin A, 80,000 IU/kg vitamin D, 200 mg/kg vitamin E, 4250 ppm Zn, 2000 ppm Mn, 700 ppm Cu, 20 ppm Co, 70 ppm Se and 16 ppm I); [6]Calculated on the basis of the protein degradability of ingredients

Table 2

Daily energy and protein balance of the two experimental groups

Energy and protein concentration	Group HD	Group LD
NE_1, MJ/kg DM	7.09	7.35
Crude protein, %DM	17.55	23.43
Rumen degradable protein, %DM	10.50	9.30
Undegradable protein, %DM	4.00	7.60

Balance*	Allowance (NRC, 1989)	HD	LD
NE_1, MJ	147.5	–5	0.2
Crude protein, g	3538	–601	–148
Rumen degradable protein, g	2146	–29	–284
Undegradable protein, g	1382	–372	136

*Calculated with a milk production of 40 kg 3.5% FCM/day

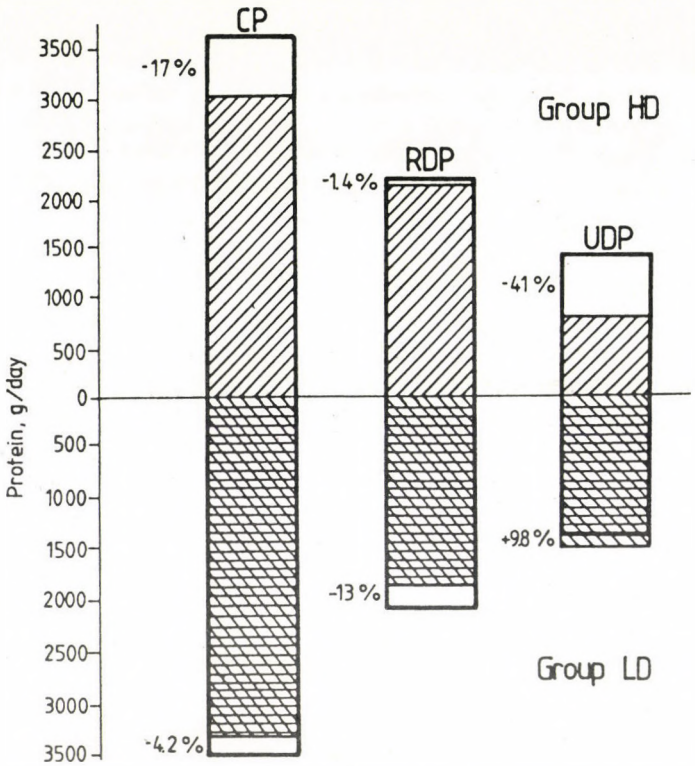


Fig. 1. Comparison of the cows' protein requirement and supply

Investigation of genital function

To assess postpartum genital functions, rectal palpation (RP) and vaginoscopy were performed when the cows were selected for the experiment in the first week after calving and then simultaneously with the other metabolic examinations. In addition, blood plasma samples were taken for progesterone (P4) assay every 3 to 4 days until the end of the feeding trial. Individual postpartum progesterone profiles were created and the duration of acyclicity as well as the time of first ovulation after calving were calculated as described earlier (Huszenicza et al., 1987, 1988*a*, 1988*c*). When all cows were supposed to have completed their uterine involution anatomically (i.e. at postpartum day 45 ± 6), they were given a $PGF_{2\alpha}$ treatment (25 mg Dinoprostum, as Enzaprost inj. ad us. vet., Chinoin, Budapest, Hungary), repeated 11 days later, to synchronise their cycle. The expected time of synchronised oestrus (i.e. 72 to 96 h after the second $PGF_{2\alpha}$ treatment) was scheduled to coincide with a RP and vaginoscopy, as well as with an assessment of metabolic status on day 59 ± 6. If (1) any clinical symptoms indicative of complications in uterine involution could be recognised at any of the checks, the cow was not included in the final evaluation. Cows showing visible oestrous signs at the time of $PGF_{2\alpha}$ synchronised oestrus or later were inseminated and their reproductive data were monitored until postpartum day 200.

Determination of body composition

Urea space was determined 5 times, early in the morning, before the first meal. A 0.9% NaCl solution containing 20% urea was administered in a volume 0.66 ml/kg liveweight (LW) into the jugular vein (Bartle et al., 1983). Twelve min after the end of infusion blood samples were collected by jugular puncture (Gad and Preston, 1990). Urea space and body fat content were calculated using the following equations:

$$US\ (\%) = \text{mg of urea-N infused/change in PUN} \times \text{liveweight} \times 10.$$
$$\text{Fat (\% of LW)} = 81.2 - 1.28\ US\ (\%)$$

where LW = liveweight, kg, PUN = plasma urinary nitrogen and US = urea space, % of LW.

Laboratory analysis of samples

Feed samples were analysed according to the A.O.A.C. (1980) procedures. Plasma samples were stored at –20 °C until the laboratory analyses. Urea nitrogen (PUN, mg/100 ml) was determined as described by Marsh et al. (1965). The intra-

and interassay coefficients of variation (CV) for PUN were 1.8 and 1.0%, respectively.

The plasma progesterone and cortisol levels were determined after prior extraction with petroleum ether (progesterone) or directly (cortisol) by ^{3}H-labelled radioimmunoassay methods, adapted by our laboratory and described in detail elsewhere (Csernus, 1981; Huszenicza et al., 1993; Thúróczy et al., 1996). The validity data of assay procedures in cows were as follow: for progesterone, sensitivity: 20.0 fmol/tube, corresponding to 0.20 nmol/l; accuracy (spiking recovery): 95%, intra-assay variation 6.9 ± 3.4%, inter-assay variation 8.0 ± 2.6%; for cortisol, sensitivity: 11.4 fmol/tube, corresponding to 1.1 nmol/l; accuracy (spiking recovery): 103%, intra-assay variation 7.0 ± 0.4%, inter-assay variation 7.8 ± 0.9%.

Ruminal fluid samples were obtained from cows with the help of a stomach tube before the morning feeding on the last day of the experimental period. The pH was measured immediately, using a portable pH meter. Ammonia and urea concentrations of the rumen fluid were measured by the phenol-hypochlorite method (Chaney and Marbach, 1962). Total lactic acid content was determined by the photometric micromethod of Velősy (1979). Analysis of VFA was done according to the gas chromatographic procedure outlined by Supelco (1975) with an SP-1200/1% H_3PO_4 on 80/100 Chromosorb WAW column.

The concentrations of free amino acids (FAA) were determined in deproteinised plasma by ion-exchange chromatography, using a BIOTRONIK LC 5001 amino acid analyser. From the FAA concentration profile, total essential amino acids (TEAA) and total non-essential amino acids (TNEAA) were calculated. The sum of TEAA and TNEAA was the total free amino acid (TFAA) concentration (Husvéth et al., 1982).

Samples of blood serum were analysed for thyroxine (T_4) and 3,3',5-triiodothyronine (T_3) by radioimmunoassay (Pethes et al., 1985). Plasma triglyceride, total cholesterol (TCh) and high density lipoprotein cholesterol (HDL-Ch) were determined as described by Wieland and Seidel (1983) and Rayssiguier et al. (1988). Blood haemoglobin and glycohaemoglobin, as well as plasma total protein, albumin and fructosamine levels were analysed according to Bárdos and Oppel (1989) and Johnson et al. (1982). Blood glucose and plasma creatinine, non-esterified fatty acid (NEFA) and acetoacetate (ACAC) concentrations, as well as plasma aspartate aminotransferase (AST) activity were determined by the use of commercially available kits (Boehringer, Mannheim, Germany) or other widely accepted colorimetric methods (Huszenicza et al., 1988*a*, 1988*b* and 1988*c*). Cholic acid concentration was determined with a commercially available ^{125}I-labelled RIA kit (National Institute for Radiobiology, Budapest, Hungary). The design of the experiment is shown in Fig. 2.

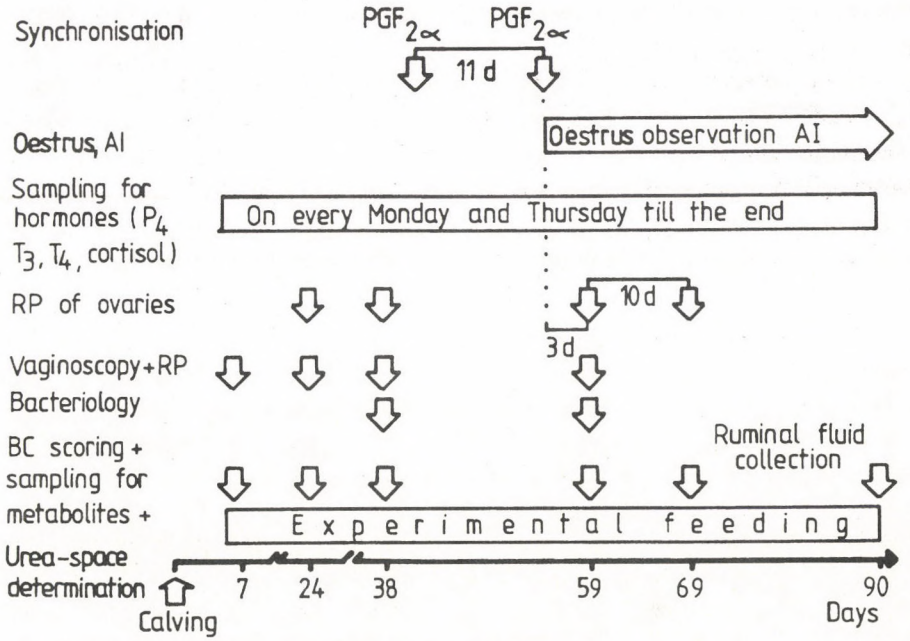


Fig. 2. Experimental design

Statistical calculations

Productive, metabolic and reproductive data, as well as the liveweight, body condition score (BCS) and urea space were subjected to analysis of variance and the calculation of non-sequential least squares means. Methods utilised for the statistical analyses included (1) Student's *t*-test (for pair-wise comparison of group means), (2) one-way analysis of variance giving the least significant difference (LSD) at the level of 5% (Kleinbaum and Kupper, 1978), for comparison of means calculated on different postpartum days, as well as (3) the general linear model procedure from SAS (Snedecor and Cochran, 1967; SAS, 1988).

The recommendations laid down in the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (1988) were strictly observed throughout the experiment.

Results

If symptoms of organic and/or metabolic diseases (mastitis, lameness, ketosis) had occurred, the cow was excluded from the evaluation. Eventually, 17 and 15 of the HD and LD cows, respectively, were included in the final analysis of data.

Milk production, body weight, condition and composition

The actual milk production was slightly higher in group LD than in group HD cows. During the first 91 days of lactation the difference was 95 kg of 3.5% fat corrected milk. The average daily difference in milk fat and milk protein production was 87 and 65 grams, respectively, in favour of group LD.

Although a decrease in body condition score (BCS) and body weight could be detected in both groups, compared to the body weight measured just after calving the changes found 5 weeks later were significant ($P < 0.05$) only in the LD animals. In week 10, the BCS and the body weight of HD cows were again the same as recorded within 7 days of calving. In group LD, however, the reduction remained obvious in both parameters until the end of experimental feeding (Table 3). Initially the urea space of the two groups did not differ. However, during the trial as a whole, the urea space of LD cows was continuously larger ($P > 0.05$) and the calculated body fat content lower than in HD animals.

Metabolic adaptation

All metabolic parameters remained within the physiological limits in both groups and none of the cows proved to be ketotic in week 3 or later. As a result of a more increased long-lasting mobilisation of body reserves at the second and third samplings, significantly ($P < 0.05$ to 0.01) higher NEFA levels were observed in LD cows than in those receiving the HD diet (239 ± 90 and 158 ± 50 vs. 137 ± 20 and 111 ± 10 μmol/l in groups LD vs. HD, respectively).

The actual blood glucose levels and both of the parameters reflecting the long-term tendencies of glucose concentration (fructosamine and glycohaemoglobin percentage) were uniformly low in the first week after calving. Later on the glucose and fructosamine levels obviously rose in cows of both dietary groups, with no differences between the groups. By day 24 ± 6 (glucose) or day 38 ± 6 (fructosamine), these values reached a plateau significantly ($P < 0.001$) higher than the values measured just after calving. Compared to the values found immediately after calving, the postpartum elevation in glyco-Hgb levels became significant ($P < 0.05$ to 0.01) on day 38 ± 6 in HD cows but only about one month later in animals kept on LD diet. A usual postpartum increase with a plateau at and after the 4th sampling on day 59 ± 6 was found in TCh and HDL-Ch levels which tended to be higher in HD cows from the 2nd sampling on day 24 ± 6. Since the same sampling a clear-cut tendency of higher blood urea levels could be demonstrated, which was noteworthy even if the between-group differences were significant ($P < 0.05$ to 0.001) only in the 2nd and the last samples. No further diet-dependent tendencies could be observed in haemoglobin, creatinine, total protein, albumin, ACAC, cholic acid and triglyceride concentrations, AST activity or packed cell volume (PCV).

Table 3

Time- and diet-dependent differences in body condition score (BCS), body mass, urea space and body fat (mean ± pooled SEM)

Feeding group		Postpartum day of sampling (± 6 d)					F	LSD
		< 7	24	38	59	69	($p < 0.05$ values)	
BCS	HD	2.71 ± 0.17	2.59 ± 0.16	2.44 ± 0.15	2.38 ± 0.18	2.51 ± 0.17	0.22	1.54
	LD	2.77 ± 0.17	2.45 ± 0.10	2.43 ± 0.12	2.32 ± 0.13	2.35 ± 0.14	1.78	0.38
	SEM	0.17	0.13	0.14	0.17	0.16		
Body mass, kg	HD	591 ± 21	579 ± 19	576 ± 19	574 ± 20	592 ± 28	0.15	75
	LD	598 ± 17	560 ± 15	553 ± 14	557 ± 14	563 ± 14	1.51	42
	SEM	19	17	17	17	21		
Urea space, %	HD	49.0 ± 1.3	50.0 ± 2.1	51.1 ± 1.5	51.6 ± 1.7	50.6 ± 1.9	2.45	4.45
	LD	48.7 ± 1.5	51.0 ± 1.9	51.2 ± 1.3	52.1 ± 1.8	51.8 ± 2.1	2.62	3.94
	SEM	1.39	1.98	1.40	1.73	1.00		
Body fat, %	HD	18.44 ± 0.51	17.27 ± 0.73	15.80 ± 0.46	15.16 ± 0.51	16.39 ± 0.61	2.49	1.49
	LD	18.90 ± 0.57	15.89 ± 0.57	15.73 ± 0.39	14.54 ± 0.49	14.92 ± 0.59	2.35	1.27
	SEM	0.54	0.65	0.43	0.50	0.60		
Body fat, kg	HD	109 ± 2.96	100 ± 4.17	91 ± 2.67	87 ± 2.86	97 ± 3.61	2.45	8.47
	LD	113 ± 3.36	89 ± 3.25	87 ± 2.19	81 ± 2.74	84 ± 3.38	2.34	7.13
	SEM	3.16	3.71	2.43	2.80	3.50		

In group HD: n = 17; in group LD: n = 15

As part of the metabolic adaptation, a slight increase in T_3 and an obvious elevation in T_4 levels were detected simultaneously with advancing lactation. Clear tendencies of lower T_3 and higher T_4 concentrations were found in group LD during the whole experimental feeding period, reaching a level of significant ($P < 0.05$ to 0.01) differences only around the expected time of the first postpartum ovulation (Fig. 3). Postpartal changes in cortisol concentrations were less characteristic, with the only obvious between-group differences on days just before the expected time of synchronised oestrus. During the synchronisation procedure the cortisol levels were significantly ($P < 0.05$) more elevated in the LD cows (Fig. 3).

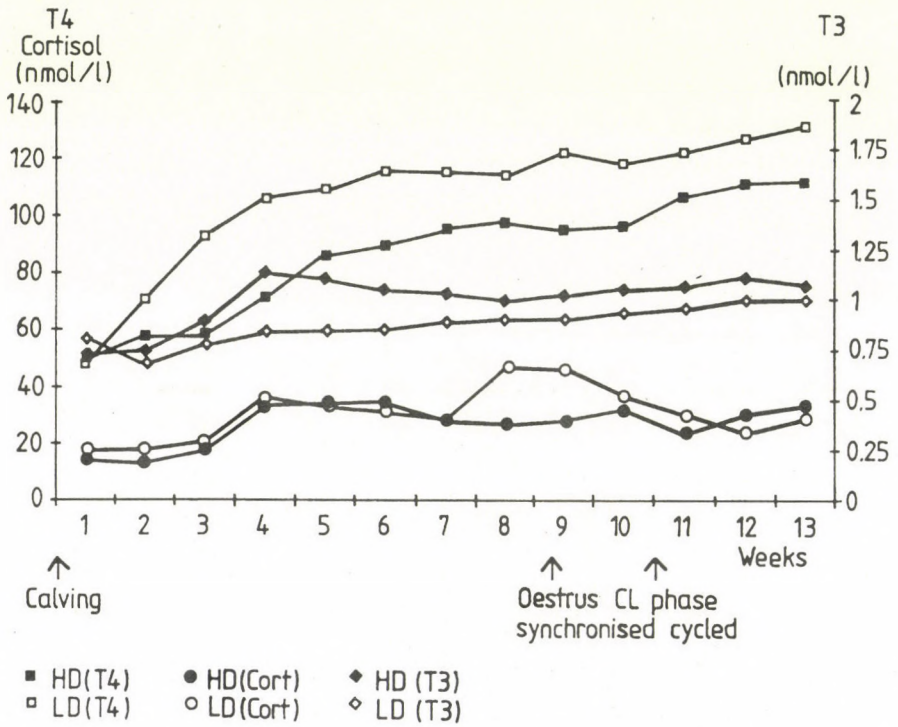


Fig. 3. Cortisol, thyroxine (T4) and triiodothyronine (T3) levels of HD and LD cows

Ruminal response. Amino acid and lipid composition in blood

At the end of the experimental feeding period the pH of the rumen fluid of HD cows was higher than that of LD animals. The ammonia and urea concentrations of the ruminal fluid of HD cows exceeded those of cows of group LD. There was practically no difference in the lactic acid level. The C2:C3 ratio was 2.7:1 vs. 2.3:1 in groups HD and LD, respectively.

All the detected plasma amino acids but cystine showed significant fluctuations at different times after parturition. Total free amino acid (TFAA) concentration was significantly ($P < 0.001$) higher at postpartum week 2 than at parturition. Subsequently, it declined in week 4 ($P < 0.01$) only to undergo another significant ($P < 0.001$) rise in week 8. The concentrations of alanine, serine, arginine, histidine, lysine, phenylalanine, threonine and tyrosine showed changes comparable to those found in TFAA concentration. Nevertheless, the levels of isoleucine, leucine and methionine were significantly lower 2 and 4 weeks post partum than immediately after calving. HD and LD rations caused the biggest differences in plasma TFAA concentration 4 weeks after parturition. The plasma TFAA level of cows of group LD exceeded that of HD animals by about 0.2 mmol/l (Fig. 4). This increase was primarily due to the significantly higher leucine, tyrosine and phenylalanine concentrations of LD cows. Unlike these amino acids, the plasma concentration of isoleucine was significantly lower in group LD. No significant difference was found ($P > 0.05$) between groups HD and LD in the plasma concentration of the other amino acids. At the same time, the free amino acid profile (Fig. 5) and blood lipid composition did differ, reflecting the dietary treatments (Fig. 6).

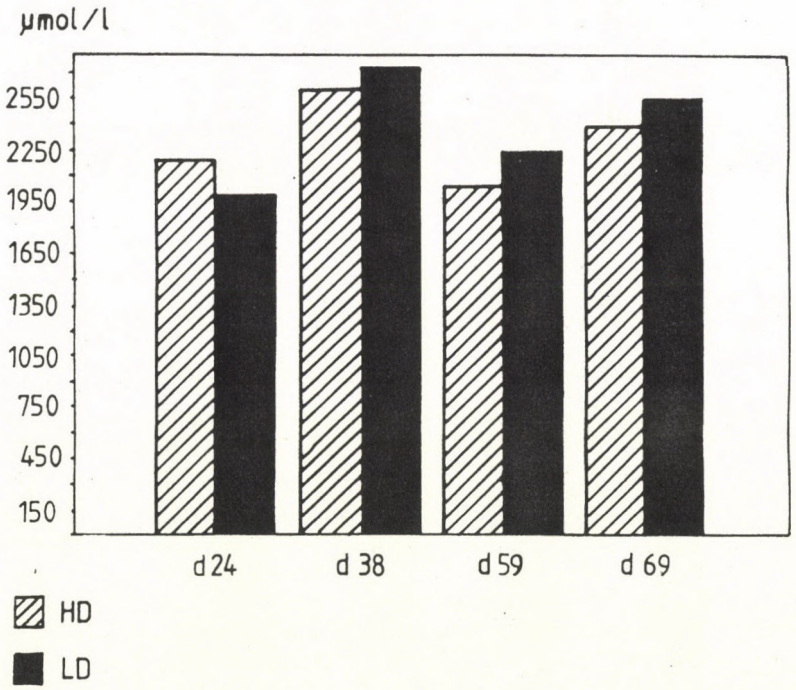


Fig. 4. Total free amino acid concentrations in the blood

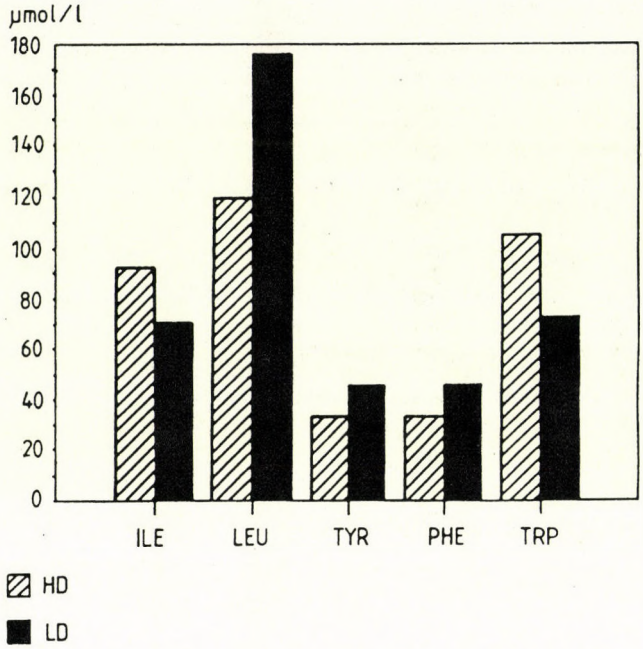


Fig. 5. Differences in the branched-chain amino acid concentrations of the blood in HD and LD cows

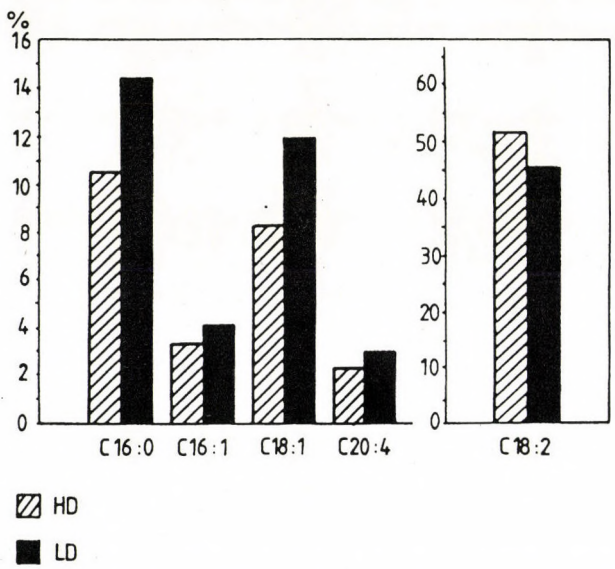


Fig. 6. Lipid profile of the blood of cows in the two experimental groups

Postpartum ovarian function, $PGF_{2\alpha}$-synchronised heat and reproductive performance

Based on the individual postpartum progesterone profiles, the cows kept on an LD diet usually ovulated slightly later and with more obvious individual variation than those receiving the HD diet. The mean duration of postpartum (pp) acyclicity were 32.4 ± 22.3 and 26.7 ± 15.9 days in groups LD and HD, respectively. Three cows in group LD and one cow in group HD remained acyclic for 60 days or longer. After the second administration of $PGF_{2\alpha}$ only 2 LD but 11 HD cows ($P < 0.05$) showed visible oestrous symptoms and could be inseminated (AI). In the subsequent period the mean progesterone levels of cows with synchronised cycles were within the physiological limits and close to each other; therefore, no more details are given here in that concern. Based on post-AI progesterone profiles, during the implantation period embryonic mortality could be suspected in 1 LD and 2 HD cows. None of the cows in group LD and 5 cows in group HD conceived as a result of AI performed at the time of $PGF_{2\alpha}$-synchronised oestrus. Naturally, later on some additional animals conceived in both groups. There was no difference between the groups in pregnancy rate within 200 days after calving, but cows of group LD showed their first visible oestrus and reconceived significantly ($p < 0.05$) later (Table 4).

Table 4

Ovarian function* and reproductive performance during the first 200 days after calving (means ± pooled SEM)

Parameter	Group HD	Group LD	SEM
Number of cows	17	15	
Postpartum acyclia, day	26.7[a]	32.4[a]	4.80
$PGF_{2\alpha}$-insensitive CL	2	6	
acyclic	1	3	
early developing CL	1	3	
$PGF_{2\alpha}$-sensitive CL (>5 d)	15	9	
After 2nd $PGF_{2\alpha}$ treatment			
no answer**	3	3	
incomplete luteolysis	1	4	
visible oestrus + AI	11	2	
First visible oestrus	64.4[a]	82.9[b]	5.76
Calving to reconception, days	80.1[a]	118.2[b]	8.74

*based on the individual P4 patterns; **based on the individual P4 profiles and clinical investigations; [a,b]Means in a row with different superscripts differ significantly ($P < 0.05$)

Discussion

There are several reports in the literature which deal with the effect of protein supply on milk production and/or reproduction of high-yielding dairy cows in the postpartum period; however, only a few of these studies are characterised by a complex approach.

Body condition generally decreases during early lactation, increases from the peak of lactation, and is constant during the dry period. Efficiently producing dairy cows maintain a mean score of 2.5 throughout the lactation cycle. Wildman et al. (1982) found that the BCS of high-producing dairy cows was between 2.47 and 2.60 in the first 80 days of lactation. Based upon the results of Otto et al. (1991), one unit of change in BCS is associated with a 56 kg change in liveweight. Wright and Russel (1984) estimated that a one-unit change in BCS corresponds to a 7% change in body water. The correlations between body condition score and chemically determined body fat were all very highly significant. One unit in condition score was associated with a change of 52.6 to 84.1 kg of fat. In our case, one unit of BCS corresponded to 87.6 kg of fat, calculated on the basis of the urea space.

Despite the fact that urea space is generally proportional to empty body water, a considerable variation has been noted. For instance, Bartle et al. (1988) stated that urea dilution accounts for only 60 to 70% of the variation in body components. In their study the range of urea space of sheep was between 40.0 to 69.6% of liveweight, with standard deviations between 4.8 to 7.6. The above authors explain the observed variation by individual differences in urea clearance, the low number of samples (two samples taken at minutes 0 and 12) used in that method, and possible technical errors in the body weight and plasma urea nitrogen determinations. The imprecise sampling time can also be a source of variation. In the present study the standard variation in urea space was between 5.0 to 8.1%, which is similar to values reported in the relevant literature. On the basis of this study we agree with the statement of Bartle et al. (1983), that "with this degree of precision, comparison of body composition based on urea space measurements would best be made between groups of cows. Urea space may also be useful for measuring changes in body composition in individual cows during changes in nutritional or physiological state."

Satter and Slyter (1974) studied the effect of ammonia concentration on microbial protein and volatile fatty acid production. They found that under nitrogen-limiting conditions the microbial protein yield and total acid production increased with supplementary urea, reaching a plateau at 50 mg NH_3-N/l (3.6 mmol/l). Roffler and Satter (1975) postulated 5 mg ammonia nitrogen/100 ml (3.6 mmol/l) rumen fluid as the ruminal ammonia concentration. In our case, NH_3-N concen-

tration reached the suggested value in the LD animals (3.79 mmol/l = 52.6 mg/l) and exceeded that in cows of group HD (5.76 mmol/l = 80.6 mg/l).

Feeding a diet of (too) low rumen degradability under N-limiting conditions may have reduced the quantity of bacterial protein and the efficiency of fibre degradation. The present data suggest a higher optimum of ruminal NH_3-N level than stated by Roffler and Satter (1975). It seems that the range set by Verité et al. (1979) for maximum organic matter digestion (5.7 to 10.7 mmol NH_3-N/l) is more appropriate for high-producing dairy cows in early lactation. More efficient utilisation of fibre by HD animals was shown by the different acetic to propionic acid ratio (2.7 vs. 2.3).

Feeding high escape CP (i.e. LD) sources decreased plasma glucose and urea N (Rusche et al., 1993). We did not find differences between the groups in blood glucose level. Owing to the intake of excess rumen degradable protein, the plasma urea nitrogen (PUN) levels were elevated (from 10.2 to 14.8 mg/dl = 3.64 to 5.29 mmol/l) in group HD throughout the experiment (Elrod and Butler, 1993). The present data also show a higher urea-N concentration in the blood plasma of HD cows but, at the same time, they indicate that the values found by us were not due to a protein surplus.

Energy deficit may be detrimental to fertility through its effect on hypothalamic and ovarian function. It may be accompanied by a marginally deficient intake of several vitamins and minerals, which together may be detrimental to fertility. It seems that in high-yielding dairy cows the mammary gland takes priority over the reproductive system. *In vivo* measurements of body composition (D_2O) showed that the shortest duration of the anovulatory period (DAP), in days (i.e. 36 days), was observed for dairy ewes which gained 1 kg body lipids during the first month post partum. Above and below that threshold, the DAP increased. There were good correlations between the duration of the anovulatory period and changes in body lipid but not in protein (Bocquier et al., 1993). Canfield and Butler (1991) observed a significant correlation between cumulative (or average) energy balance and the duration of anoestrus after calving.

An increasing amount of evidence indicates that excessive protein intake during early lactation may be detrimental to postpartum fertility. Protein solubility and degradability are also important in that respect.

Soderman et al. (1987) proved that administration of exogenous gonadotropin-releasing hormone (GnRH) increased serum progesterone during the luteal phase in cows fed a 15% CP diet but not in cows fed a 22% CP diet. Plasma progesterone was higher on day 15 of the oestrous cycle in cows fed a 12% CP diet than in cows fed 23% CP (Jordan and Swanson, 1979; Swanson, 1989). However, as Ferguson and Chalupa (1989) have emphasised, the solubility and degradability of dietary protein can be important sources of variation (Wearer, 1987).

Several studies demonstrated altered LH secretion, decreased progesterone concentrations, decreased conception rates and increased average days open when high (16 to 23 per cent) protein diets were fed in early lactation (Ferguson, 1986).

Blood plasma urea nitrogen level may be a useful indicator of rumen degradable protein intake. Serum urea N levels higher than 20 mg/dl (7.17 mmol/l) may be associated with decreased fertility (Ferguson, 1986). In Finnish studies, too low levels (below 2.5 mmol/l) of urea during the puerperium were associated with poor fertility (Miettinen, 1991).

Low HD protein supply decreases ration digestibility and available energy. In contrast, increased crude protein in the ration increases digestibility of dry matter (fibre) and available energy, and minimises body condition loss (Ferguson, 1986).

Recent studies by Gibb and Iving (1993) have reinforced the point that LW and BCS offer an inexpensive and reasonably accurate means of estimating body composition.

Negative energy balance reduces the secretion of progesterone, and it was found to be negatively correlated with NEFA (Villa-Godoy et al., 1990). FFA concentrations were highest in cows calving at the highest body condition score (BCS = 4). Milk fat from cows calving at BCS 3 was characterised by the highest content of short-chain fatty acids and the lowest content of long-chain and unsaturated fatty acids (Perdon et al., 1993).

Negative energy balance and the rate of mobilisation of body reserves in early lactation are directly related to the postpartum interval to first ovulation and to a lower conception rate (Butler and Smith, 1987), as was found also in the present study. In the trial of Staples et al. (1990), anoestrous cows lost more body weight and had a more negative energy status than did cycling cows.

Bruckental et al. (1989) found that, at a satisfactory degradable protein supply, fish meal supplementation improved the reproductive traits. Blood urea reflected the amount of rumen degradable CP, independently of the total CP intake (Table 5). Serum urea N levels below 7 mg/dl and above 20 mg/dl (Ferguson, 1986; Miettinen, 1991) may be associated with decreased fertility.

It can be concluded from the results of Roseler et al. (1993) and the present study that the lack of undegradable protein has a negative influence on milk production (Table 5). The PUN primarily reflects the urea fermentation potential (UFP) of the ration rather than the crude/degradable protein intake (Burroughs et al., 1984; Magdus et al., 1988).

In the study of Sonderman and Larson (1989), the blood progesterone levels were lower in cows fed 20% than in those receiving 14% CP on day 12 of both the oestrous cycles synchronised with exogenous GnRH and those occurring subsequently.

Table 5

Milk yield and plasma urea N concentrations (means) by diet (Roseler et al., 1993; present study)

Diet	DIP[1]:	UIP	CP	Milk kg/day	[2]PUN mmol/l	MUN mmol/l	UFP g
A	90	87	90	23.6	2.93	2.00	–35.7
B	112	112	112	26.4	5.29	4.14	–107.1
C	140	100	110	24.4	5.89	4.86	–131.0
D	104	150	122	25.2	6.36	5.14	–140.4
E	150	112	135	26.0	7.39	6.36	–181.0
HD	99	87	83	36.2	5.91	4.71[3]	–126.0
LD	73	110	96	37.7	5.07	4.00[3]	–102.4

[1] DIP = degradable intake protein, UIP = undegradable intake protein and CP = crude protein as a percentage of NRC (1989) requirement; [2]PUN = blood plasma urea nitrogen; MUN = milk urea nitrogen; UFP = urea fermentation potential (Burrough et al., 1984), calculated according to Magdus et al. (1988); [3]Calculated after Roseler et al. (1993)

Rasby et al. (1991) stated a relationship between the BCS and the serum concentration of T_4, i.e. their beef cows had lower (64 ng/ml) serum concentration than cows in moderate body condition and fat animals (72–73 ng/ml).

Rayssiguier et al. (1988) indicated that the beginning of lactation was associated with a low concentration of VLDL and LDL, and that these concentrations were inversely related to the fat content of the liver. Total and HDL-cholesterol tended to be reduced in LD cows, which can partly explain the poorer fertility of this group (Grummer and Caroll, 1988).

As a conclusion of the present study, one can state that differences between the experimental diets were reflected in the characteristics of the ruminal fluid, the amino acid and lipid profiles of the blood plasma, as well as in urea concentration.

Milk production can be stimulated by feeding more bypass/escape protein. However, combined with a marginal energy supply this higher milk yield causes a longer energy deficit. Increased energy deficiency can be shown in changes of the body weight, BCS, urea space, as well as through the concentrations of NEFA, TCh, HDL-Ch, and glycohaemoglobin after calving. It should be emphasised that the changes found in this study remained in the physiological range.

The same statement is valid for the dynamics of T_3 and T_4 levels of the blood (Fig. 3). The interrelationship between thyroid function and digestion is well known (Kennedy et al., 1977), but in this case the measurement of hormone concentrations was not sensitive enough to elucidate the functional status of the thy-

roid gland. One would have to use TRH stimulation and/or measurement of the kinetics of T_3-rT_3 transformation (Pethes et al., 1985).

The higher cortisol levels measurable at about days 50–60 in group LD (Fig. 3) may be attributed to the energy deficit. All of the described phenomena may be explained by the dissimilar homeorrhetic regulation of milk production and reproduction (Bauman and Currie, 1980). The glucose, fructosamine, Hgb, total protein, triglyceride, albumin and cholic acid concentrations of the blood are under strict endocrinological and homeostatic regulation (Kaneko, 1989). Therefore, these parameters could not detect any possible distinct physiological differences between the HD and LD cows.

Concentration of urea in the blood plasma has been used as an index of nitrogen status in ruminants (Preston et al., 1965). In our case, the higher ruminal NH_3 level of group HD cows was reflected in an increase of blood urea concentration. Similarly, Rusche et al. (1993) found that the feeding of high escape CP sources lowered plasma urea concentration.

The similarities of NEFA concentrations reflect the small differences between treatments in BCS change (Pedron et al., 1993; Villa-Godoy, 1990).

However, the increased lactose synthesis for milk could result in a reduction of blood glucose concentration. In the present case, the difference between the two groups in milk production was too small to elicit this phenomenon.

In the rumen of HD cows the pH and the NH_3-urea concentration were higher. These were reflected in a higher blood plasma urea level, too (Table 5).

The bigger amount of RDP supposedly improved the N supply of bacteria (including the cellulolytic ones), which can be proved by the shift in the acetate to propionate ratio. The more efficient utilisation of fibre in group HD can partly explain the more favourable energetic and subsequent reproductive status of HD animals.

The free amino acid profile and the lipid composition of the blood plasma (Figs 4, 5 and 6) reflected the dietary treatments: as a result of the characteristically high leucine and phenylalanine content of blood meal and the fatty acids from fish meal (C16:0, C16:1, C18:1, C20:4), these proved to be higher in LD cows.

The higher total free amino acid concentration found in group LD (Fig. 4), showed that the amino acid supply of LD cows at tissue level was better than that of HD animals. The extra amino acid set must have been derived from the feed, as protein catabolism/degradation in the tissues are minimal (Andrew et al., 1995). This could contribute to the higher milk production of LD cows.

Reproduction

Recently it has become widely accepted that in non-suckling, multiparous dairy cows, being over the nadir of postpartum energy imbalance, cyclic ovarian function resumes within 10 days, and that there is a correlation between postpartum weight loss and the duration of acyclicity (Butler et al., 1981; Henriksen and Jensen, 1985). The duration, rather than the momentary depth, of energy imbalance was reported to influence the resumption of cyclicity (Huszenicza et al., 1988*c*). Not only the time of the first ovulation but also that of the first visible oestrous symptoms are affected by the long-lasting postpartum energy deficiency (Harrison et al., 1989). As reflected in the metabolic parameters detailed above, the cows fed an LD diet were forced to mobilise their body reserves for a longer period and more intensively than those of group HD. Since the delaying effect exerted by bacterial complications of uterine involution on the onset of cyclic ovarian function (Fonseca et al., 1983; Huszenicza et al., 1987) did not appear in this study, these slight metabolic differences could result in distinct between-group differences in postpartum acyclicity and in the ratio of cows with visible oestrous symptoms, i.e. suitable for AI 72–96 h after the second $PGF_{2\alpha}$ treatment. Based on these differences, clear-cut tendencies of a lower success rate of AI performed at $PGF_{2\alpha}$-synchronised oestrus, a longer interval between calving and first visible heat, and a longer calving-to-conception interval could be demonstrated in the LD cows.

To sum up the findings of the present study, it can be concluded (Fig. 1) that in high-producing dairy cows a moderate deficiency of rumen degradable protein sources during the first 10 weeks after calving (group LD) can be more detrimental to ovarian function and reproductive performance than a severe deficiency of undegradable protein (group HD). That deficiency exerts its negative effect by influencing rumen function and, first of all, energy metabolism. Therefore, in the case of an RDP deficit, the benefits of feeding excess UDP to dairy cattle to maintain peak milk production should always be compared with its potential negative effects on fertility.

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Abbreviation key

ACAC = acetoacetate
AI = artificial insemination
ANOVA = analysis of variance
AST = aspartate aminotransferase
BCS = body condition score
CL = corpus luteum
CLI = corpus luteum insuffitient
CP = crude protein
d = day
FCM = fat corrected milk
FFA = free fatty acid
h = hour
HD = high degradable
HDL = high density lipoprotein
LD = low degradable
LDL = low density lipoprotein
LW = liveweight
NEFA = non-esterified fatty acids
NRC = National Research Council
P4 = progesterone
PCV = packed cell volume, haematocrit
$PGF_{2\alpha}$ = prostaglandin $F_{2\alpha}$
pp = post partum
PUN = plasma urinary nitrogen
RDP = rumen degradable protein
RIA = radioimmunoassay
RP = rectal palpation
SEM = Standard Error of the Mean
T_3 = triiodothyronine
T_4 = thyroxine
TCh = total cholesterol
TEAA = total essential amino acid
TMR = total mixed ratio
UDP = undegradable protein
US = urea space
VFA = volatile fatty acid

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AN INVESTIGATION ON PLASMA PROGESTERONE LEVELS DURING PREGNANCY AND AT PARTURITION IN THE IVESI SHEEP

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In this study, plasma progesterone concentrations in a total of 45 Ivesi sheep were investigated during pregnancy and at parturition. Blood samples were collected from all the sheep studied every 20 days commencing on the 30th day of pregnancy. Plasma progesterone levels were determined by radioimmunoassay. During the second half of the pregnancy, progesterone concentrations in the sheep giving birth to one, two and three lambs were 2.02 ± 0.08 ng/ml, 3.24 ± 1.18 ng/ml and 4.90 ± 0.85 ng/ml, respectively. There was no significant variation in progesterone levels between sheep with male and female offspring. At parturition, plasma progesterone concentrations did not differ significantly between the animals. The present results indicate that plasma progesterone levels could be used not only to determine the pregnancy status of the animal but also to predict the number of fetuses after the second half of the pregnancy.

Key words: Sheep, progesterone, pregnancy, fetuses, parturition

The presence of progesterone hormone in serum is required for the maintenance of pregnancy in animals (Izhar et al., 1992; Silver, 1994). Progesterone follows a regular pattern during the oestrous cycle and during pregnancy and declines in the prepartum period and at parturition in sheep (Smith, 1982; Silver, 1994). Therefore, determination of plasma progesterone gives important clues about the reproductive activities of animals. There have been several reports in the literature on the progesterone levels of sheep (Emady et al., 1974; Weigl et al., 1975; Boulfekhar and Brudieux, 1980; Smith, 1982; Hamon and Heap, 1990; Silver, 1994). Hamon and Heap (1990) reported that progesterone first slightly increased during the early part of pregnancy. They also noted that following this

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slight increase, there was a reduction between gestation days 30 and 50. Afterwards, the progesterone levels elevated again (Hamon and Heap, 1990). Emady et al. (1974) demonstrated that a quantitative assay of plasma progesterone could be used for predicting the number of fetuses carried by the ewes. However, this conclusion has not been shared by other workers (Weigl et al., 1975; Chauhan et al., 1991).

In the present study plasma progesterone levels were investigated throughout pregnancy and at parturition. The objectives of the study were to utilise progesterone levels as a criterion in determining the number and sex of fetuses. The influence, if any, of the number and sex of the fetus on plasma progesterone levels was also investigated.

Materials and methods

A total of 45 Ivesi sheep were employed. During the month of September, when most sheep experience oestrus, rams were left with the ewes for a period of 15 days. Thirty days after ram exposure, blood samples were collected from the jugular veins into heparinized containers at 20-day intervals until and at parturition. The samples were centrifuged at 3000 rpm for 20 min at 4 °C. Plasma samples were separated and stored at –20 °C until assayed.

Plasma progesterone levels were determined by radioimmunoassay (RIA). RIA reagents were purchased from Diagnostik Systems Laboratories Inc. (Webster, Texas, USA). The method has previously been described (Yalow and Berson, 1971). The inter- and intra-assay coefficients of variation were 10.8% and 9.5%, respectively. The sensitivity of the assay was 15 pg/tube.

The data were statistically analysed by Student's *t*-test. The level of significance was set at $P < 0.05$.

Results and discussion

As shown in Fig. 1, during pregnancy mean ± SEM plasma progesterone concentrations were 2.02 ± 0.08 ng/ml, 3.24 ± 1.18 ng/ml and 4.90 ± 0.85 ng/ml in the ewes giving birth to one, two and three lambs, respectively.

Statistical analysis of these results showed that the differences between the test groups were significant. The changes between plasma progesterone concentrations of the sheep giving birth to male and female lambs were, however, not significant (Fig. 2). This analysis was performed only on the ewes with one lamb.

Plasma progesterone levels were also measured at parturition in all the animals studied. However, these concentrations did not seem to be in-

fluenced by the number and sex of the fetus. At parturition, plasma progesterone concentrations were found to be less than 1 ng/ml for all of the ewes (Table 1).

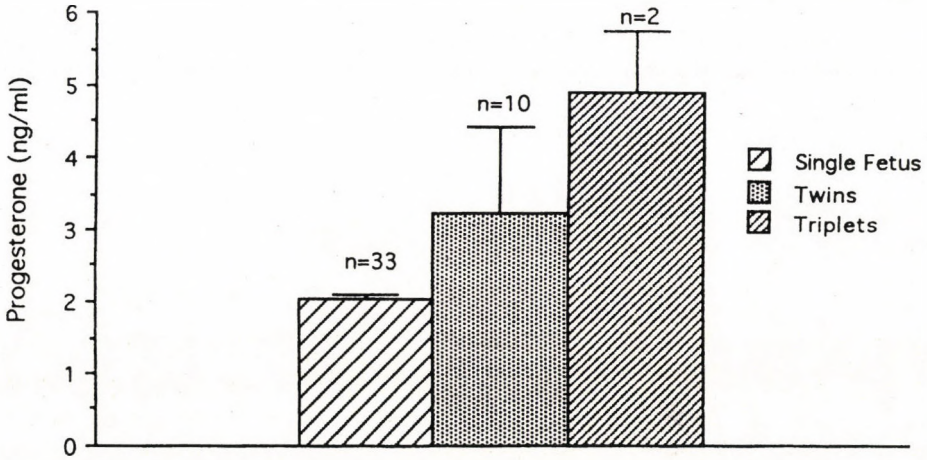


Fig. 1. Mean ± SEM plasma progesterone concentrations (blood samples collected at 20-day intervals) in the Ivesi sheep during pregnancy according to the number of fetuses carried

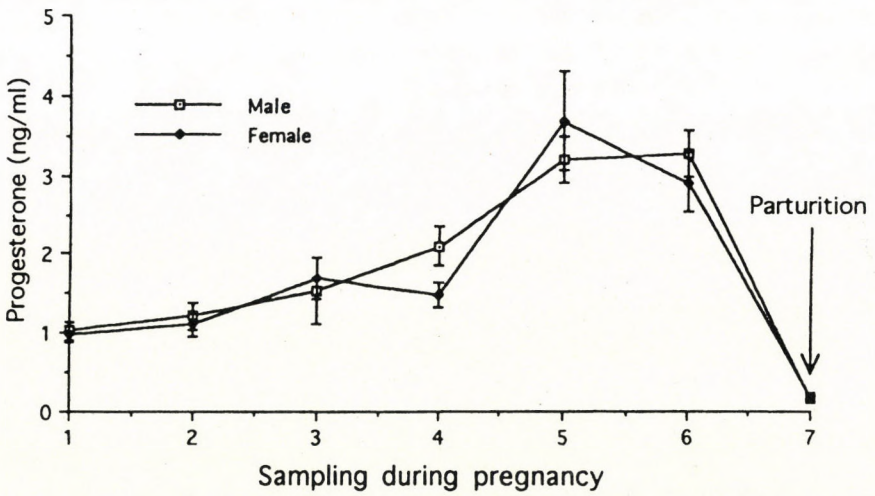


Fig. 2. Mean ± SEM plasma progesterone concentrations (blood samples collected at 20-day intervals) in the Ivesi sheep according to the sex of the fetus

Table 1

Plasma progesterone concentrations (ng/ml) during pregnancy and at parturition in Ivesi ewes giving birth to one, two and three lambs

Sample date	I	II	III			
	Single fetus n = 33	Twins n = 10	Triplets n = 2	t I–II	t I–III	t II–III
(1) 24.11.1993 (~ 30 days)	1.00±0.08	1.16±0.17	1.43±0.32	–	–	–
(2) 15.12.1993	1.16±0.11	1.97±0.29	2.96±0.54	P≤0.005	P≤0.005	–
(3) 05.01.1994	1.61±0.26	1.99±0.15	3.58±0.83	–	P≤0.05	P≤0.005
(4) 02.02.0994	1.81±0.16	3.39±0.49	4.85±0.45	P≤0.0005	P≤0.0005	–
(5) 24.02.1994	3.42±0.31	5.77±0.54	7.60±0.90	P≤0.0005	P≤0.005	–
(6) 09.03.1994	3.12±0.23	5.18±0.55	9.00±0.80	P≤0.0005	P≤0.0005	P≤0.01
(7) Parturition	0.18±0.05	0.12±0.01	0.17±0.07	–	–	–

n: number of animals in each group, t: Student's *t*-test

Overall, these results show that there is a very close relationship between plasma progesterone concentrations and the number of fetuses the animals carry ($P < 0.001$; Fig. 3).

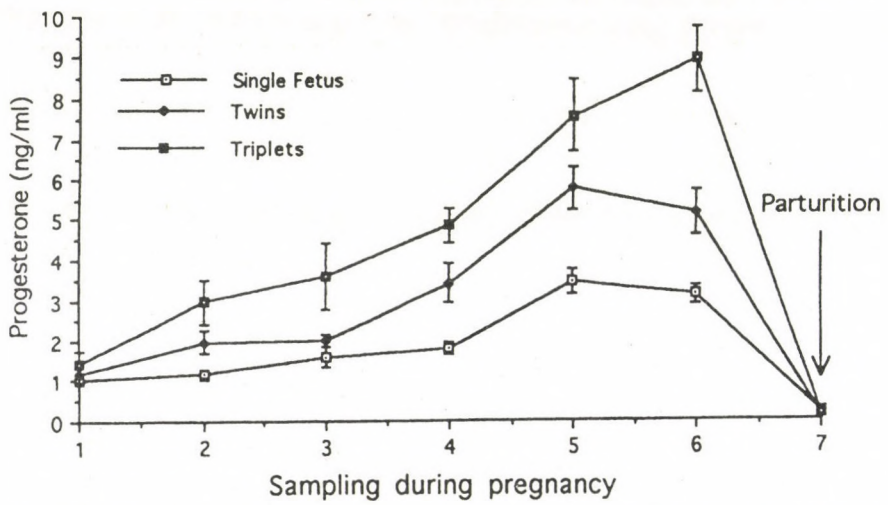


Fig. 3. Mean ± SEM plasma progesterone concentrations in the Ivesi sheep during pregnancy (blood samples collected at 20-day intervals) and at parturition

Discussion

In this study, the critical importance of plasma progesterone for the maintenance of pregnancy has been confirmed once again.

The number of fetuses and the stage of pregnancy seem to influence the plasma progesterone concentrations dramatically. These findings indicate that, in addition to the corpus luteum, placental tissues are also involved in the synthesis of progesterone. It is likely that the corpus luteum is not able to synthesize enough progesterone to maintain pregnancy in cases where more than one fetus is present. In such instances, it is possible that placental tissues synthesize more progesterone than the corpus luteum. Recently it has been shown that the presence of a corpus luteum is not required for maintenance of the latter part of pregnancy in sheep (Silver, 1994).

There have been conflicting reports on the relationship between plasma progesterone levels and the number of fetuses the ewes carry (Emady et al., 1974; Weigl et al., 1975; Hamon and Heap, 1990; Chauhan et al., 1991).

Emady et al. (1974) reported that irrespective of the number of fetuses, serum progesterone concentrations in sheep did not differ during the first month of pregnancy. However, between the first and the second month the difference in serum progesterone concentrations started to be noticed. In their study, mean ± SEM hormone levels at the second month of pregnancy were 1.73 ± 0.26 ng/ml, 2.31 ± 0.40 ng/ml, and 3.27 ± 0.89 ng/ml in sheep giving birth to one, two and three lambs, respectively (Emady et al., 1974). Between days 110 and 130 of pregnancy these values reached 3.78 ± 1.45 ng/ml, 5.09 ± 0.43 ng/ml, and 9.18 ± 1.26 ng/ml, respectively. Hamon and Heap (1990) also found that plasma progesterone concentrations in sheep giving birth to only one lamb were lower than those with two or three lambs.

In this study, it was noticed that during the first month of pregnancy, the mean ± SEM plasma progesterone concentrations were 1.00 ± 0.08 ng/ml, 1.16 ± 0.17 ng/ml and 1.43 ± 0.32 ng/ml in sheep with one, two and three offspring. These values during the last month of pregnancy were 3.12 ± 0.23 ng/ml, 5.18 ± 0.55 ng/ml, and 9.00 ± 0.80 ng/ml, respectively. There was a positive relationship between the number of fetuses and mean plasma progesterone concentrations ($P < 0.001$). These findings show that plasma progesterone concentrations could be used to ascertain the number of offspring the animal will produce.

Plasma progesterone concentrations at parturition in all animals were found to be less than 1 ng/ml. These results are consistent with those published by other workers (Emady et al., 1974; Tsang, 1978; Boulfekhar and Brudieux, 1980; Hamon and Heap, 1990; Shipka and Ford, 1991).

In this study, it was also observed that the sex of the fetus did not seem to influence plasma progesterone levels (Fig. 3). Consequently, plasma progesterone concentrations do not appear to be a useful tool in predicting the sex of the fetus.

In conclusion, the present results indicate that plasma progesterone levels in the sheep could be used not only to determine the pregnancy status of the animal but also to predict the number of fetuses after the second half of pregnancy.

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EXPERIMENTAL TRANSDUODENAL DIATHERMIC SPHINCTEROTOMY IN PIGS

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Transduodenal diathermic sphincterotomy was performed in nine pigs of the Large White breed, weighing 70–80 kg. The Erbotom T 400 diathermy unit equipped with a needle knife was used to incise the duodenal papilla and its sphincter. The animals were divided into three groups and the results of the operation were estimated 2, 8 and 12 weeks after surgery. After the respective survival period was over, the animals were examined post mortem to macroscopically inspect the surgery site and take samples for histopathological and histochemical investigations. Histopathological preparations were stained with haematoxylin and eosin as well as by Hale-Müller's or Frankel's methods. Histochemical investigations were performed to determine the activity of three enzymes: alkaline phosphatase, acid phosphatase and succinate dehydrogenase. The present study has revealed that diathermic incision of the duodenal papilla and its sphincter gives very good results as confirmed by the findings of macroscopic as well as histopathological and histochemical investigations.

Key words: Duodenal papilla, sphincterotomy, diathermy, pig

Transduodenal sphincterotomy or sphincteroplasty is an operation on the duodenal papilla that improves the outflow of bile and pancreatic juice in different disease entities.

The first operation on the duodenal papilla was performed in 1891, by McBurney, who incised the papilla to remove a wedged gallstone. In 1895, Kocher described a method for removing concrements from the terminal part of the bile tract through the incision of the duodenal wall and common bile duct. After removing the concrements, he sutured the common bile duct to the duodenum, creating the suprapapillary ducto-duodenal connection. In 1919, Archibald introduced transduodenal sphincterotomy for the surgical treatment of pancreatitis. Transduodenal sphincteroplasty was first performed in a case of recurrent pancreatitis (Jones and Smith, 1952), then it was used in the treatment of biliary and

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pancreatic diseases (Jones et al., 1969). In the 'seventies, German surgeons (Classen and Demling, 1974; Classen and Safrany, 1975) and a Japanese surgeon (Kawai et al., 1974) introduced endoscopic incision of the duodenal papilla, a technique which subsequently underwent tremendous development and gained widespread use.

Modern surgery possesses some methods with regard to the duodenal papilla and the hepato-pancreatic ampulla, including techniques for incision of the papilla (papillotomy), incision of the sphincters (sphincterotomy), plastic repair of the hepato-pancreatic sphincter (sphincteroplasty), and endoscopic sphincterotomy.

Putting aside the technical differences between these operations, generally two ways of reaching the duodenal papilla can be distinguished. The first is classical laparotomy and duodenotomy, and the second is exposure of the papilla by endoscopy of the alimentary tract using modern fiberoptic techniques. Their common feature is the final intended effect of improving the outflow of bile and pancreatic juice through the terminal part of the bile-pancreatic tract (Doubilet, 1958; Safrany and Cotton, 1981; Dowsett et al., 1988; Siegel et al., 1988; Ghazi and Washington, 1989; Simon et al., 1989).

This study was aimed at investigating the usefulness of the diathermy knife in transduodenal sphincterotomy. The use of the knife allowed us to omit a very arduous and precise stage of the sphincteroplasty, i.e. the creation of mucous membrane adhesion with surgical sutures. Bleeding, which is otherwise a very frequent complication, seldom occurred when the diathermy knife was used.

Materials and methods

The operations were performed on nine pigs of the Large White breed, weighing 70–80 kg. After 24-h fasting, 30 min before surgery the animals were given 0.03 mg/kg of atropine (Polfa, Poland) s.c. and 0.4 mg/kg of propionylpromazine (Combelen, Bayer, Germany) i.m. The main anaesthetic was pentobarbital (Vetbutal, Biowet, Poland) given at a dose of 10–20 mg/kg i.v. The animal was laid on its left side. The abdominal cavity was opened with an about 20 cm long incision of the abdominal wall along the right costal arch. The duodenum was opened with a longitudinal, 4 cm long incision, starting 1 cm behind the pylorus. After the duodenal papilla was localized (usually 2–3 cm from the pylorus), the catheter was inserted into its opening and the papilla was incised with the diathermy knife (the incision was 10 mm long). The German diathermy unit Erbotom T400 was used for incising the duodenal papilla and its sphincter. The diathermy knife was a needle, which enabled precise incision of the sphincter. The power of

the unit was always set at the 5th grade of the scale. The duodenum was closed longitudinally, with two-layered dexon sutures. Finally the incision of the abdominal integument was closed with common layered sutures. On the day after surgery the animals were fed one-fourth of the daily dose of the protein feed prepared as liquid fodder. During the following days the daily doses of feed were gradually increased to reach the full dose on day 7 or 8 after surgery.

The pigs were divided into three groups and the results of the operations were assessed at 2, 8 and 12 weeks after surgery. All animals were subjected to physical examinations including measurement of the body temperature, pulse and respiration rate daily in the first six days after surgery, and then once a week until the end of the survival period. After the respective survival period was over, the animals were examined post mortem and tissue samples comprising a segment of the duodenum with the common bile duct were taken. Then the duodenum was cut open, the operated region was visualised and examined macroscopically for length of the papilla and sphincter postoperative scar, the presence of inflammatory reaction, and progression of the adhesion of the duodenal and common bile duct mucous membranes. Subsequently the common bile duct was cut along the surgical incision and opened to complete the macroscopic observations. After the macroscopic examinations were completed, the preparation was divided into two parts by cutting the papilla and common bile duct longitudinally. The part intended for histopathological investigations was fixed by immersion in 10% neutral formalin, while that designed for histochemical investigations was frozen and kept at –20 °C.

Histopathological preparations comprised sites of adhesion of duodenal and bile duct mucous membranes. These preparations were stained by three methods: (1) routine haematoxylin and eosin staining; (2) Hale-Müller's method as modified by Ritter-Oleson to study mucopolysaccharides; (3) Frankel's method to investigate collagen fibres.

Histochemical investigations were performed to determine the activity of three enzymes: (1) alkaline phosphatase, using Gomori's precipitation method; incubation periods were 1 or 3 h at 37 °C, pH 9.6; control reaction was performed; (2) acid phosphatase, using Gomori's precipitation method; incubation periods were 1 or 3 h at 37 °C, pH 5.5; control reaction was performed; and (3) succinate dehydrogenase (SDH), using the method of Nachlas; incubation period was 15 min at 37 °C.

Results and discussion

Clinical examinations of all the animals during the particular experimental periods did not reveal disease symptoms, and the values of body temperature, pulse and respiration rate were in the reference range. The animals had good ap-

petite, no clinical symptoms of dyspepsia, and by weeks 8 and 12 after surgery they reached a body mass of 100–110 kg or 120–130 kg, respectively.

The first parameter estimated at postmortem examination was the length of the papilla and the sphincter postoperative scar. In three pigs, the scar length was found to be the same as during the surgery, but in six animals it was 1 mm shorter.

The next parameter investigated was the presence of an inflammatory reaction in the region of the papilla and sphincter incision made with the diathermy knife. No macroscopic signs of inflammatory reaction (thickening or deformations) were found in any of the animals, and the line of adhesion was practically invisible. The presence of inflammatory reaction is thought to be related to the degree of adhesion of the duodenal and common bile duct mucous membranes. The adhesion was considered to be complete when the transition zone between the mucous membranes was invisible. Such a macroscopic picture was found in all animals.

The minor (up to 10%) shortening observed in the length of incision in 6 operated pigs may be related to the normal healing of tissues cut with the diathermy knife. The absence of inflammatory reaction and cicatrization signs in the region of the incision as well as the complete adhesion of the mucous membranes indicated that the bile and pancreatic tract opening was maintained broad and patent (Fig. 1).

Histopathological examination revealed that the structure of the duodenal and bile duct mucous membranes, their adhesion and the neighbouring tissues were normal in six pigs. In the remaining animals, the histological structure of the mucous membranes was abnormal. In one of these pigs, signs of inflammation were related to bacterial complication rather than to the surgical procedure.

In the tissue specimens prepared 2 weeks after surgery, no signs of inflammatory reaction were found, the adhesion of mucous membranes was complete and only small morphological changes could be observed. The glandular layer was well structured, and collagenization was considered to be delicate and normal. Mucopolysaccharides were stained to assess the structure of the mucous membrane and myxopoiesis. With the method used, the mucopolysaccharides assumed a purple colour. The glandular layer was found to be well developed (Fig. 2).

At 8 and 12 weeks after surgery, histology revealed no signs of inflammatory reaction. In all cases there was complete adhesion of the mucous membranes and normal collagenization of moderate intensity (Fig. 3). Mature collagen fibres assumed dark green colour, while immature ones were light green and the reticulin fibres were brown. The intensity of collagenization in the operated region is one of the most important parameters indicating the ability of the incised tissue to undergo cicatrization.

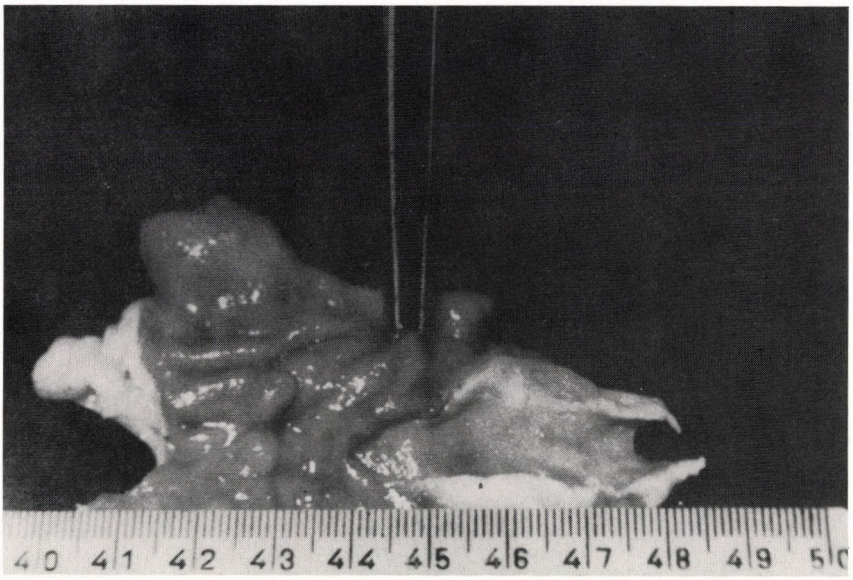


Fig. 1. The operated site 12 weeks after surgery. Note the incised papilla and the wall of the bile duct

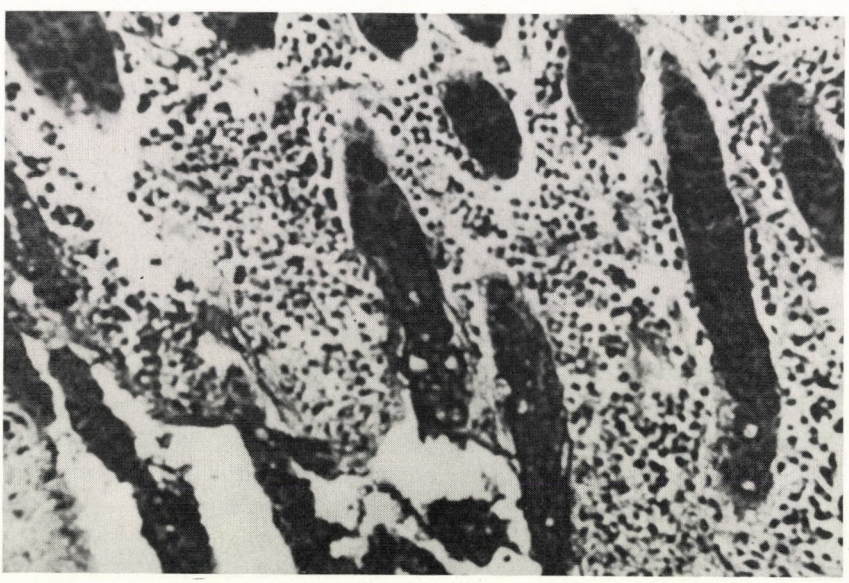


Fig. 2. Site of the adhesion of duodenal and bile duct mucous membranes with the glandular layer 12 weeks after surgery. Mucopolysaccharide staining, × 180

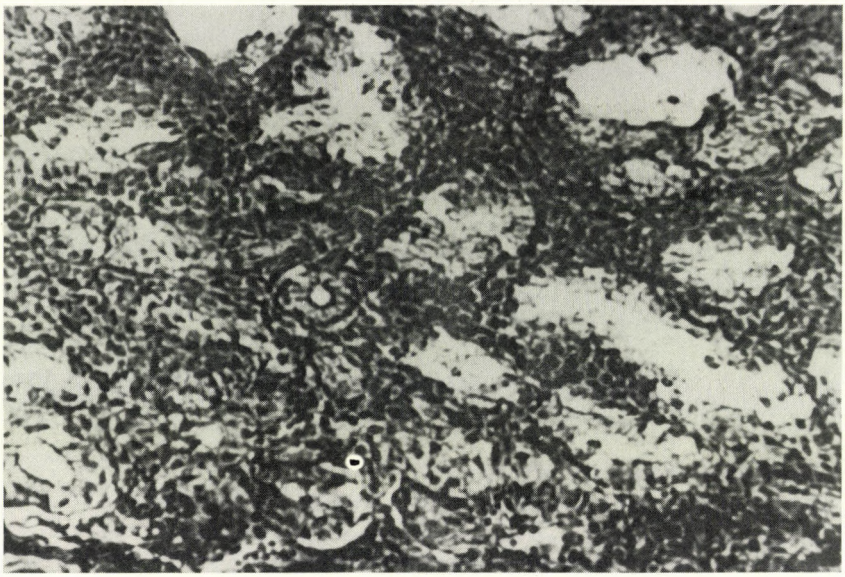


Fig. 3. Collagenization of moderate intensity 12 weeks after surgery. Collagen staining, × 180

Alkaline phosphatase is an enzyme occurring mainly within the cells of the connective tissue. It can also be found in the duodenal mucosa. The level of alkaline phosphatase influences the functioning of cells of the phagocyte system, e.g. that of some macrophages. In the present study, the activity of this enzyme was investigated to estimate the intensity of connective tissue proliferation in the operated region, to assess the activity of cells infiltrating this area and the appearance of the glandular layer of the mucous membrane. The activity of alkaline phosphatase in the mucous membranes of the duodenum and common bile duct was similar in all animals. The differences found in the site of mucous membrane adhesion were related to connective tissue generation. The activity and type of the histochemical reactions of alkaline phosphatase can indicate the degree of tissue injury. Injuries cause an increase in enzyme activity. Three types of histochemical reaction can be recognized: the granular reaction typical of intact cells, the diffusive reaction typical of injured cells, and the intermediate, granular-diffusive reaction, which is very difficult to estimate. An increased activity of acid phosphatase was found in tissue specimens prepared 2 weeks after surgery, and the type of histochemical reaction was considered to be the granular-diffusive one. Eight and 12 weeks after surgery, similar features of enzyme activity, accompanied by the granular-diffusive reaction, were found in three animals. In the remaining pigs

only the granular reaction was observed. No differences were demonstrable in the activity of acid phosphatase between the duodenum and the common bile duct.

Succinate dehydrogenase (SDH) is one of the important respiratory enzymes, located within mitochondria. Its activity is indicative of respiratory processes in cells. Therefore, the activity of this enzyme can be related to the level of metabolic processes in the respective tissue. The activity of SDH decreased in histological specimens prepared from the duodenal mucous membrane 2 weeks after surgery. Its activity increased parallel to restitution of the epithelium and glands in the duodenal mucous membrane. In the histological specimens prepared 8 and 12 weeks after surgery, SDH activity was high in all glandular cells (Fig. 4).

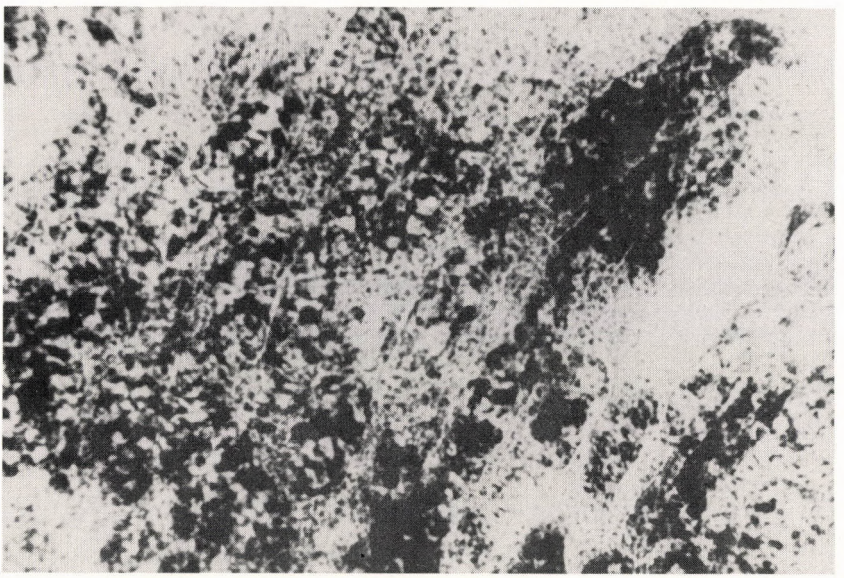


Fig. 4. The region of mucous membrane adhesion 12 weeks after surgery. Succinate dehydrogenase (SDH) staining, × 60

The present study has demonstrated that diathermic incision of the duodenal papilla gives very good results, as confirmed by the findings of macroscopic as well as histopathological and histochemical examinations.

Classical sphincteroplasty usually produces an inflammatory reaction leading to cicatrization, stenosis and deformation of the operated region, which is related to incision of tissues with the scalpel, suturing of mucous membranes, and the presence of sutures within the tissue for a longer period.

This study has shown that diathermic sphincterotomy results in a complete adhesion of the duodenal and common bile duct mucous membranes, without any signs of inflammatory reaction and stenosis of the operated region. The results of histological and histochemical investigations have confirmed these observations.

The practical benefits of diathermic sphincterotomy over classical sphincterotomy include precision of incision, diathermic haemostasis, shortened time of operation, and lack of cicatricial scar. In addition, it makes the use of mucosal sutures unnecessary.

In veterinary surgery, diathermic sphincterotomy can be used for removing concrements from the terminal part of the bile duct and in cases of persistent anatomical stenosis.

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STUDIES ON END-TO-END COLONIC ANASTOMOSIS IN THE DOG: A COMPARISON OF TECHNIQUES

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For end-to-end anastomosis, many techniques suitable for the small intestine end up catastrophically when applied on the colon. An experimental study involving 18 healthy adult dogs was conducted to find a model technique out of the best considered techniques for small intestinal end-to-end anastomosis viz., simple interrupted approximating sutures (group I), double-layer inverting sutures (group II), and Gambee sutures (group III). The results depicted a non-significant difference among groups as far as paralytic ileus and omental adhesions are concerned. Similarly, haematologic and physiologic values did not deviate from a normal pattern after any surgical intervention. Barium sulphate radiographs taken at postoperation (PO) day 14 revealed significantly lesser reduction ($P \leq 0.001$) in lumen diameter ($26.14 \pm 1.87\%$) at the site of anastomosis in group I as compared to $30.16 \pm 1.20\%$ and $38.91 \pm 1.87\%$ reduction in groups III and II, respectively. Similarly, gain in tensile strength was maximum ($26.55 \pm 1.33\%$) in group I and minimum ($19.73 \pm 2.62\%$) in group II on PO day 14. The current study showed superiority of the simple interrupted suture technique for colonic end-to-end anastomosis over the other two techniques studied.

Key words: Anastomosis, end-to-end, suture techniques, colon, dog

Diseases of the gastrointestinal tract amenable to surgical correction are fairly common in dogs (Tangner, 1982). Many of these necessitate resection of the affected portion of the gut and re-establishment of its continuity by anastomosis. Among problems commonly encountered with intestinal anastomosis, leakage at the anastomotic site in the large bowel has a far greater devastating potential and is more likely to overwhelm normal body defences than in small bowel anastomosis (Nichols et al., 1973). Moreover, colonic anastomosis, owing to the relatively tenuous blood supply and decreased ability to lay down collagen in comparison to that in small intestine, poses a vexing problem (Hawley, 1973). The optimal suture pattern for performing colonic anastomosis is still a matter of controversy. The objective of the current research project was to evaluate the comparative efficacy of three anastomotic techniques on the colon of the dog.

*The submitted paper is part of the principal author's M.Sc. (Hons.) thesis.

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Materials and methods

Experimental animals

A total of 18 adult healthy mongrel dogs of both sexes were randomly divided into 3 groups of 6 animals each. A thorough clinical examination including physiological parameters such as body temperature, pulse, and respiration rates, and laboratory examinations on blood, faeces and urine (Coles, 1984) was conducted during a 7-day acclimatization period for baseline data.

Preoperative preparations and anaesthesia

Prior to surgical intervention, food and water were withheld for 18 and 6 h, respectively. All the dogs were premedicated with atropine sulphate at 0.045 mg/kg body weight (b.w.) subcutaneously and acepromazine maleate (Sedastress®, Farvet, Holland) at 0.25 mg/kg b.w. intramuscularly, 30 and 15 min prior to surgery, respectively. General anaesthesia was induced and maintained with a 5 per cent solution of thiopentone sodium (Pentothal sodium, Abbott Laboratories, Pakistan) intravenously, to effect.

Anastomosis techniques

The standard caudal coeliotomy and resection procedures were the same for every surgical manoeuvre in this experiment (Grier, 1975). The end-to-end colonic anastomosis technique used in dogs of group I was simple interrupted approximating sutures (DeHoff et al., 1973), in group II it was double-layer inverting sutures (Halsted, 1891), whereas in dogs of group III Gambee sutures (Gambee, 1951) were placed (Figs 1a, b and c).

Postoperative management and observation

Water was offered *ad libitum* followed by milk on PO day 2 and a soft diet on PO day 3. Monitoring of the animals consisted of recording the physiological parameters daily up to PO day 14. Similarly, haematological parameters were recorded on alternate days till PO day 13. Time for return of defaecation was also recorded.

All the dogs were euthanized on PO day 14 and processed for the evaluation of perianastomotic adhesions, stenotic index, and gain in tensile strength of the operated segment of the colon. The development of adhesions was graded as 25%, 50%, 75% or 100% depending on the extent of circumference involved (Lavy et al., 1987). For stenotic index (per cent stenosis of lumen), a 12 cm long colonic segment including the anastomotic site was resected and gravity filled with

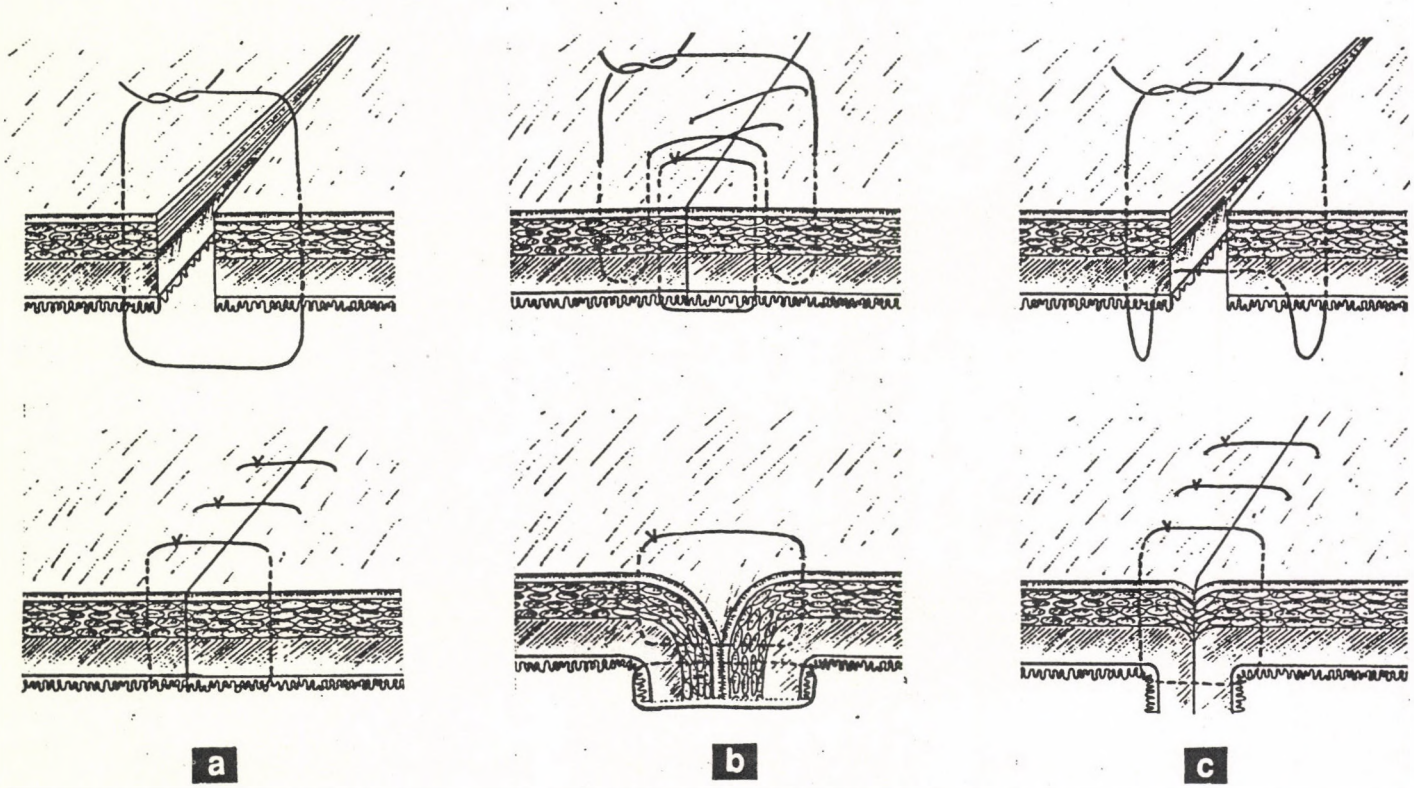


Fig. 1. Schematic representation of (a) simple interrupted approximating sutures (group I); (b) double-layer inverting sutures (group II); (c) Gambee sutures (group III)

barium sulphate enema. The stenotic indices were assessed radiographically according to McAdams et al. (1970) by the following formula:

$$\text{Stenotic index (\%)} = 100\,[1 - \{2a / (b + c)\}],$$

where a = lumen diameter (cm) at the anastomotic site,
b = lumen diameter (cm) 2 cm proximal to the anastomotic site, and
c = lumen diameter (cm) 2 cm distal to the anastomotic site.

The tensile strength (TS) of the anastomotic site was recorded with Schopper's Tensile Strength Tester No. 114-SC type (Yasuda Seiki Seisakusho, Japan; Booth, 1968). Following washing of the specimen with normal saline, three 1 × 2 cm colonic strips containing the anastomotic site in the centre and three same sized strips from the unoperated part of colon (control) were cut and evaluated for TS. The per cent gain in TS (using means of 3) was calculated by the following formula:

$$\text{Gain in TS (\%)} = \frac{\text{Mean TS of strips with anastomotic site}}{\text{Mean TS of control strips}} \times 100$$

The data were analyzed statistically using completely randomized two- and three-factorial designs and Duncan's Multiple Range test (Steel and Torrie, 1984).

Results and discussion

Intestinal surgery has undergone a lot of trials and tribulations in recent times. The principles for resection and anastomosis are similar throughout the intestinal tract. Variations occur in technique only because of inherent differences in the flora, lumen size, and healing properties of various segments. Anastomotic dehiscence has been found to be more common in the rectum and colon than in the stomach and small intestine (Goligher et al., 1977). Because of the high bacterial population, surgery of colon carries a higher risk of sepsis than that of other parts of the intestinal tract (Nichols et al., 1973). Decreased ability to lay down collagen in the colon is also an important factor in colonic surgery (Hawley, 1973).

In the present study, all the anastomotic techniques were clinically successful. The postoperative (PO) physiologic and haematologic values demonstrated a normal stress response to surgical insult or mild self-limiting infection (Coles, 1984). The post-surgical leukocytic response (19,000–21,000/mm^3) was desirable and indicative of good host resistance.

The time for normal passage of faeces, ranging from 57.33 ± 6.02 to 62.67 ± 6.02 h PO, showed a non-significant difference ($P \geq 0.05$) among the three groups. This is in agreement with the findings of Orr (1969) and Mausya et al. (1984), and suggestive of absence of any pre- or postoperative faecal contamination of the peritoneal cavity (Richardson et al., 1982; Scott-Corner and Scher, 1987).

There were comparatively lesser adhesions in the double-layer inverting technique (79.2 ± 18.8%), followed by the simple interrupted technique (87.5 ± 5.6%) and the Gambee technique (91.7 ± 12.9%). These adhesions differed non-significantly ($P \geq 0.05$) among the three groups. The development of adhesions was confined to the anastomotic site in all the dogs. The adhesions primarily involved the adjacent loop of small intestine (Fig. 2). Other structures included greater omentum, urinary bladder and uterine horn (invariably in all females) (Fig. 3). Peritonitis was not evident in any of the dogs of all groups indicating the absence of leakage or preoperative contamination of the peritoneal cavity (Scott-Corner and Scher, 1987). The higher incidence ($P \geq 0.05$) of adhesions in the Gambee technique is due to increased manipulation during its application (Conolly and Stephens, 1968).

The barium contrast radiographic study revealed a highly significant decrease in lumen diameter of all groups at PO day 14. The mean stenotic index was significantly lower ($P \leq 0.01$) in group I (26.14 ± 1.87%), followed by group III (30.16 ± 1.20%), and group II (38.91 ± 1.87%). A significantly more expressed stenosis observed after using the double-layer inverting technique (Fig. 4) as compared to the approximating technique is consistent with the findings of Hamilton (1967) and McAdams et al. (1970). Stenosis at the anastomotic site can cause complications by impeding intestinal contents, increasing intestinal pressure, thus resulting in leakage or dehiscence (Richardson et al., 1982). However, the marked degree of stenosis that developed in the double-layer inverting technique caused no observable complications such as obstipation or constipation during the study period.

The gain in tensile strength (TS) at PO day 14 was maximum in group I (26.55 ± 1.33%), followed by group III (26.05 ± 0.73%), and it was minimum in group II (19.73 ± 2.62%). Gain in TS in group II differed significantly ($P \leq 0.01$) from that in groups I and III. This criterion indicated how rapidly normal intestinal strength returned following a particular anastomotic technique (Ellison, 1981). The inverting method provides the best sero-muscular apposition, but intraluminally, it is conducive to more inflammation and collagenase activity than are other methods (Grier, 1975). The results are well supported by the findings of Letwin and Williams (1967) and Hamilton (1967).

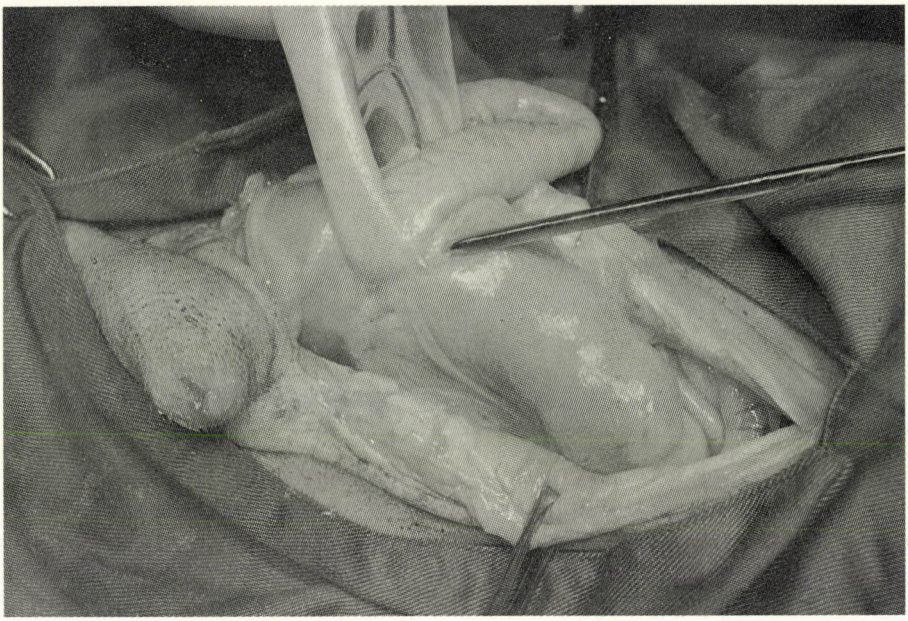


Fig. 2. Loop of small intestine adhered to the anastomotic site on colon

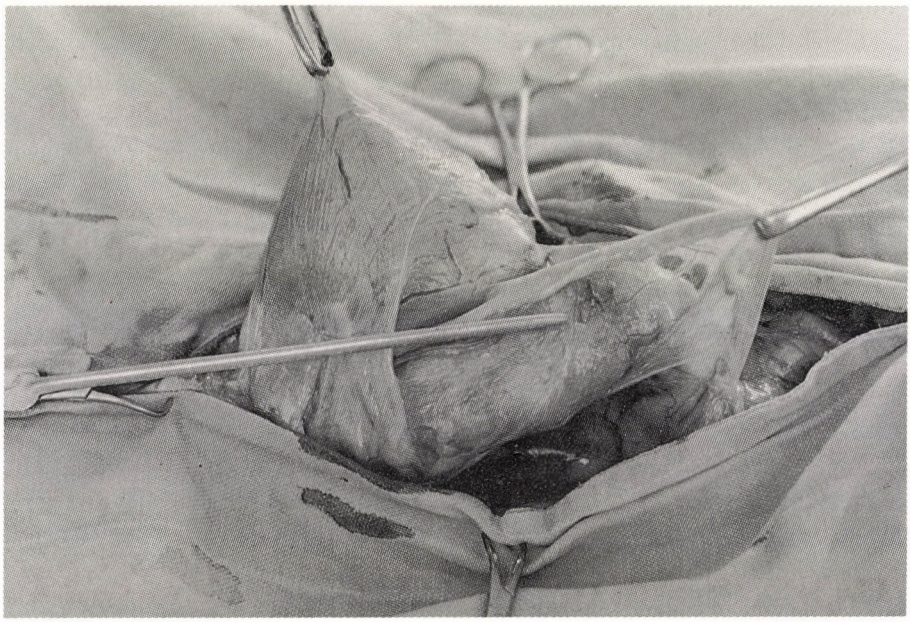
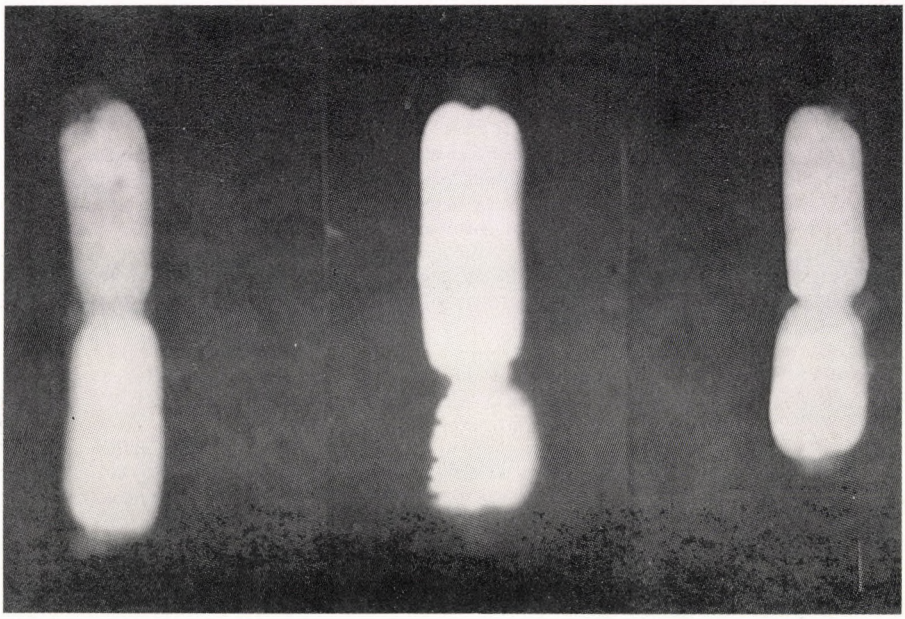


Fig. 3. Multiple adhesions between colon, urinary bladder, and uterine horn in a female dog



a b c

Fig. 4. Stenosis at the site of anastomosis in (a) simple interrupted approximating sutures (group I); (b) double-layer inverting sutures (group II); (c) Gambee sutures (group III)

From a pragmatic standpoint, the simple interrupted approximating technique satisfied the criteria for simplicity, speed and safety and is biologically sound, as it resulted in relatively lesser adhesions, minimal lumen compromise and a rapid gain in tensile strength.

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CLINICAL PERSPECTIVES OF INTRAVENOUS KETAMINE ANAESTHESIA IN PEAFOWL *(PAVO CRISTATUS)*

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A total of 29 peafowl *(Pavo cristatus)*, rectified surgically for infraorbital abscesses (n = 22), lacerated wounds (n = 4), and fractures of tibia (n = 2) and radius (n = 1), were anaesthetized by the intravenous administration of ketamine hydrochloride (Inj. Calypsol, Gedeon Richter, Hungary) in a dose of 15 20 mg/kg body weight. Divided doses (10 mg + 5 mg + 5 mg) were used with an interval of 1–2 min. No premedication was undertaken in any of the birds. Anaesthesia lasted for about 15 min and the birds gained their feet completely after 30 min to 3 hours. The respiration rate was markedly depressed (8–10/min) and the respiratory pattern was deep abdominal. Only a slight increase was observed in the heart rate. Analgesia was incomplete and muscle relaxation was not satisfactory. Mild salivation was also noticed in some of the birds (n = 3). Recovery, although not smooth, was uneventful.

Key words: Avian anaesthesia, anaesthesia, peafowl, ketamine, surgery

Birds are the most commonly kept non-domestic animals in Pakistan and include mainly species of the orders *Psittaciformes, Passeriformes* and *Galliformes*. There are now many large collections of different species in zoological parks and private bird gardens throughout Pakistan. Of these species of birds, peafowl *(Pavo cristatus)* are most liked as fancy and pet birds. Because of the obvious differences between birds and mammals, as well as the lack of familiarity with the multitude of avian species, many veterinarians are reluctant to anaesthetise birds. However, with the increasing popularity of peafowl as pet birds, more veterinarians need the skills to cope with various surgical conditions of this bird (Linn and Gleed, 1987).

A considerable body of literature is available on general anaesthesia in animals but information regarding experimental studies on general anaesthesia in birds, especially in peafowl, is both scanty and incomplete. There is a risk in formulating generalities concerning one species on the basis of observations made in another. Consequently, anaesthesia in peafowl has remained an enigma, and with-

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out competence in this field, veterinary surgeons are seriously handicapped. This is probably the reason why veterinarians demur in conducting surgery in peafowl which are very costly birds. Keeping this in view, the present study was designed to evaluate the anaesthetic effect and safety of ketamine hydrochloride in peafowl.

Materials and methods

The study was conducted on 29 peafowl of 10 months to 3 years of age, brought for various surgical conditions to the outdoor clinics of the Department of Clinical Medicine and Surgery, Faculty of Veterinary Science, University of Agriculture, Faisalabad, during the last year. The conditions included infraorbital abscesses (n = 22), lacerated wounds (n = 4), and fractures of tibia (n = 2) and radius (n = 1). Feed and water were withheld for 8 and 4 h, respectively, prior to the induction of anaesthesia. Each bird was weighed before the initiation of the trial and was restrained securely by placing it in lateral recumbency on the surgical table.

Ketamine hydrochloride (Inj. Calypsol, Gedeon Richter, Hungary), 15–20 mg/kg body weight, was administered intravenously into the brachial (wing) vein. Divided doses (10 + 5 + 5 mg/kg body weight) were used with an interval of 1–2 min. The drug was injected slowly, because ketamine given too quickly can result in apnoea and even cardiac arrest (Salerno and Van-Tienhoven, 1975).

The depth of anaesthesia was assessed by the intensity of various reflexes i.e., palpebral reflex, corneal reflex, pupil size, mandibular tone (beak) reflex, tongue irritation reflex, pharyngeal reflex, swallowing reflex, toe-pinch reflex, hock joint flexion reflex, salivation, and lacrimation. Respiratory and heart rates were also recorded. The intensity of the reflexes was classified as 3P, 2P, 1P in the order of decreasing response. The absence of the reflex was denoted as "N". Recovery was considered complete when the bird stood up on its own. Complete recovery from anaesthesia was divided into two phases. The first phase included the time between the end of surgical anaesthesia and the return of all reflexes with an intensity of 3P. The second phase included the interval between the end of the first phase and the time when the bird regained its feet.

Results and discussion

During the last 10 years, the veterinary profession has shown an increasing interest in methods of sedation and anaesthesia in birds. This has primarily resulted from the development of new drugs suitable for this purpose and the increased demand for regular veterinary care of birds, many of which are now rare and valuable or near extinction. General anaesthesia allays apprehension, abol-

ishes pain and facilitates restraint in fractious birds in order to conduct various surgical operations.

When administered intravenously at 20 mg/kg of body weight, ketamine produced light surgical anaesthesia (stages I and II) of 15 min duration. This is in line with the findings of Boever and Wright (1975), Booth (1982), and McGrath et al. (1984). The induction was generally smooth with no excitement but manual restraint was necessary in order to avoid damage to the neck and legs. The present findings are in agreement with the results of Samour et al. (1984) who conducted such studies in birds other than peafowl.

The onset of anaesthesia was rapid just within a minute after intravenous injection. This may be ascribed to the rapid distribution of the drug in all body tissues, primarily brain, adipose tissue, liver, and lungs (Lanning and Harmel, 1975). It induces anaesthesia and amnesia by the immediate disruption (dissociation) of CNS function (Booth, 1982). During the stage of surgical anaesthesia, there was complete absence of mandibular tone, tongue irritation reflex, swallowing reflex, and pharyngeal reflex (Fig. 1). This disappearance of reflexes is due to the effect of the anaesthetic agent on the midbrain (Campbell and Lawson, 1958). The corneal and palpebral reflexes did not disappear, although the intensity of response was reduced. Similar findings were reported by Altman (1980), Hartsfield (1982), Samour et al. (1984), and Linn and Gleed (1987).

The pedal reflex (withdrawal of the foot in response to toe stimulation) became slow. The same observation was reported by Altman (1980), Hartsfield (1982), and Linn and Gleed (1987). The wing stretching reflex was also not abolished completely during the anaesthetic regime. A similar observation was made by Boever and Wright (1975), Salerno and Van-Tienhoven (1975), Altman (1980), McGrath et al. (1984), and Samour et al. (1984) who pointed out that ketamine was a poor muscle relaxant in birds. The head falling reflex (orientation of head regardless of body position, i.e. righting reflex) was abolished completely during the unconscious state.

Slight salivation was observed in the anaesthetised peafowl. Similar observations were reported by Salerno and Van-Tienhoven (1975). Moderate lachrymation was also observed during the anaesthetic regimen. This is in line with the findings of Booth (1982) who recorded the same in various species other than peafowl. The most reliable indicator of the depth of anaesthesia and the patient's well-being is the respiratory rate and character. It became slow (8–10/min) and shallower as the plane of anaesthesia deepened. Ultimately it became regular deep abdominal. Hartsfield (1982) also reported the same finding.

The recovery period lasted 30 min to 3 h. This is in close agreement with the observation of Altman (1980) who indicated that it took 30 min to 5 h for birds to stand up after the administration of ketamine.

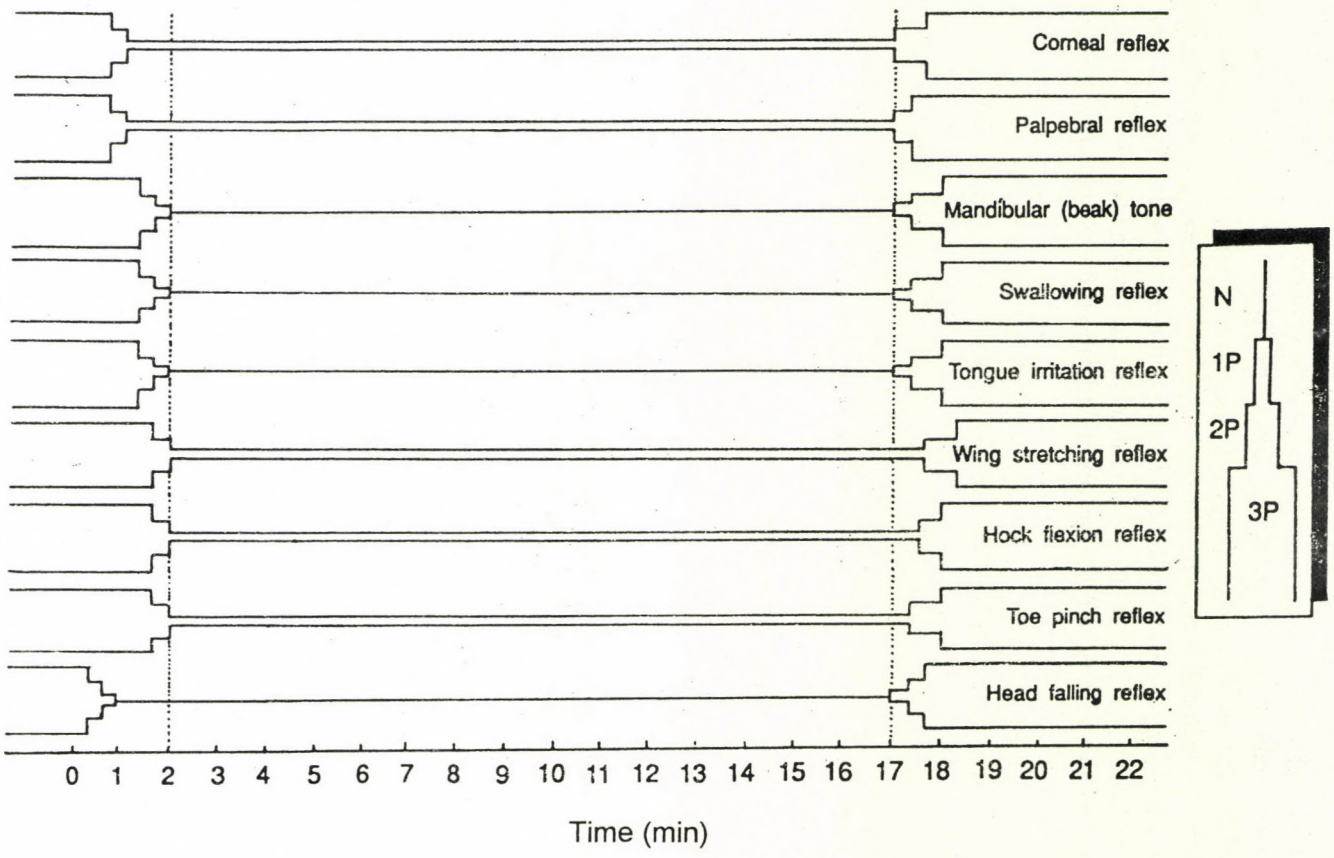


Fig. 1. Effect of ketamine (20 mg/kg b.w. intravenously) on various body reflexes of peafowl

The complications encountered with ketamine were excitement and occasional flapping of wings. The latter was much more common after pedal reflex or toe-pinch (or other painful stimulation) as well as righting reflex. Both have been reported by Mandelker (1972), Borzio (1973), Boever and Wright (1975), Amand (1977), and Green and Simpkin (1984). Cooper (1984) also reported wing flapping as a problem often encountered following ketamine anaesthesia. This can be easily controlled if the birds are securely wrapped (preferably rolled) in a strip of cloth or towel. Such restraint has the added advantage that it helps to reduce heat loss. By the time when the birds are able to clamber out of the wrapping, they will have recovered sufficiently to stand and wing flapping will be minimal. This is contrary to the findings of Richardson (1984) who never observed excitement at this stage. No fatality was observed in the present study.

Ketamine proved to be an effective and safe anaesthetic agent for the immobilisation of peafowl. The relatively complete analgesia of short duration obtained in this study persuades us to use ketamine as the only anaesthetic agent for minor surgical manipulations in birds. However, the maintenance of anaesthesia is always required in prolonged surgical procedures. Further studies should be conducted to evaluate the effectiveness and safety of ketamine in peafowl at higher dose rates.

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TOXICITY OF THE HERBICIDES FLUBALEX, FUSILADE S AND MALORAN 50 WP TO CHICKEN EMBRYOS AFTER ADMINISTRATION AS SINGLE COMPOUNDS OR IN COMBINATION

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The teratogenic effects of three herbicides (Flubalex, Fusilade S and Maloran 50 WP) were studied in chicken embryos. Each of the three test substances was administered on days 0 and 12 of incubation. Treatment was followed by evaluation on day 19. The compounds were injected into the air-chamber of eggs at three different concentrations. The medium concentration corresponded to that usually applied in chemical plant protection. In order to determine the combined toxicity of the three herbicides, the medium concentration of Maloran 50 WP and three different concentrations of Flubalex or Fusilade S each were administered simultaneously at a final volume of 0.1 ml per egg, at similar times. Evaluation was done on day 19. In tests of individual toxicity, after injection on day 0 of incubation Maloran 50 WP and Flubalex caused a significant reduction in body mass, while Maloran 50 WP and Fusilade S resulted in marked embryonic mortality. After injection on day 12, the medium and the highest concentration of Flubalex and the highest concentration of Fusilade S caused a marked increase in embryonic mortality. The developmental anomalies were of sporadic nature: their incidence increased only after Flubalex treatment, irrespective of the time of administration. The combined administration of Maloran 50 WP and Flubalex on day 0 resulted in a significant or marked body mass reduction in all groups. Embryonic mortality increased substantially after treatment with the highest dose of Flubalex, while all three concentrations of the other two herbicides led to similar results. When treatment was performed on day 12, the two highest concentrations of Flubalex and the highest concentration of Fusilade S caused expressed embryonic mortality. The developmental anomalies did not show a dose-dependent effect in any of the test series.

Key words: Flubalex, Fusilade S, Maloran 50 WP, herbicides, teratogenicity, chicken embryo, interaction

In the past 20–25 years numerous papers have been published on the ecotoxicological aspects of different pesticides. Of these works, studies on the tera-

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tological effects of such compounds in avian embryos are especially remarkable. Teratological tests have been in use for about 100 years. The first major work of this kind was done by Dareste (1891), who applied chicken embryos for teratological studies. In addition, pheasant, partridge or Japanese quail embryos are also very often used as model or non-target organisms.

Teratological tests carried out on avian embryos provide useful data for environmental protection and facilitate the development of environment-friendly chemical plant protection techniques. As teratological studies usually test the effects of a single compound at a time, the toxicological literature is extremely deficient in data on the combined toxicity of different pesticides.

In earlier studies carried out with herbicides containing 2,4-D as active ingredient, no morphological change developed after treatment on day 12 of incubation; only a reduction in embryonic body mass occurred after administration of the highest concentration (10%) (Somlyay et al., 1988).

In tests of other herbicides also containing 2,4-D as active ingredient, no deformities were observed in chicken embryos (Dunachie and Fletcher, 1967).

The objective of the experiments reported here was to obtain basic morphological data on the teratogenicity of herbicides in avian species, in addition to further information on the combined toxicity of such compounds.

Materials and methods

Test materials

Flubalex (20% benefin): liquid herbicide, practically non-toxic (acute oral LD_{50} for rats: 5,700 mg/kg).

Fusilade S (12.5% fluazifop-P-butyl): liquid herbicide, slightly toxic (acute oral LD_{50} for rats: 2,000 mg/kg).

Maloran 50 WP (50% chlorobromuron): herbicide, practically non-toxic (acute oral LD_{50} for rats: 7,100 mg/kg).

Applied concentrations (solvent: distilled water): Flubalex: 0.266, 2.66, 26.6%; Fusilade S: 0.1, 1.0, 10.0%; Maloran 50 WP: 0.1, 1.0, 10.0%. The middle concentration corresponds to that usually applied in the chemical plant protection practice. Designation of groups: control, and groups I, II and III, respectively, in the order of increasing concentration.

Methods

Eggs: Shaver Starcross 288 (Agricultural Combine of Bóly, Hungary).
Incubation: in a Ragus type incubator.

Treatment: On day 0 or day 12 of incubation 0.1 ml solution per egg final volume was administered directly into the air-chamber with an Ovijector automatic injector. Before the injection the calcic egg-shell was bored through, then, after treatment, it was sealed with paraffin (Clegg, 1964).

Processing: on day 19 of incubation.

Macroscopic evaluation: by stereomicroscopy of skeletal preparations (Dawson, 1926).

Biometric evaluation: Student's *t*-test (Finney, 1972).

Results

The numerical data obtained during the experiments are summarised in Tables 1–10.

After the administration of *Flubalex* on day 0, the average body mass of embryos significantly decreased in the groups receiving either of the two highest doses. The number of abnormally developing embryos also showed a marked increase primarily in these two groups. After Flubalex treatment on day 12, the number of embryos with developmental anomalies markedly increased in the lowest dose group, while in the groups treated with the two higher dose levels there was a conspicuous increase in embryonic mortality (Table 1).

After the administration of *Fusilade S* on day 0, the rate of embryonic mortality increased at all dose levels. If the herbicide was injected into the eggs on day 12 of incubation, a rise in embryonic mortality was observed only in the group that received the highest dose level (Table 2).

After the administration of *Maloran 50 WP* on day 0, a statistically significant or marked reduction in embryonic body mass occurred at all the three dose levels. Increased embryonic mortality was a typical finding, especially after administration of the two highest doses. After treatment on day 12, no remarkable toxic consequences were observed (Table 3).

The simultaneous administration of *Maloran 50 WP* and *Flubalex* on day 0 of incubation resulted in a significant decrease in the average body mass of embryos at all the three dose levels but especially in the group treated with the highest dose, where the rate of embryonic mortality also showed a marked increase (Table 4). After treatment on day 12, embryonic mortality increased in the group receiving the middle dose level, while the highest dose killed all the embryos treated (Table 5).

Table 1

Toxicity of Flubalex to chicken embryos after injection on days 0 and 12 of incubation

Test material and concentration (%)		Body mass (g) of embryo ($\bar{x} \pm$ S.D.)	Number of samples	Embryonic deaths (n)		Embryos showing developmental anomalies (n)	Live embryos	
				total	after treatment		number	%
Control		19.85± 1.24	20	6	6	3	14	70.0
0.26	I	19.11 ± 3.17	22	7	7	5	15	68.2
2.66	II A	18.29 ± 2.26[a]	21	5	5	6	16	76.2
26.6	III	18.17 ± 2.71[a]	27	9	9	7	18	66.7
Control		17.61 ± 2.13	26	9	6	3	17	65.4
0.26	I	17.88 ± 3.67	25	3	2	9	22	88.0
2.66	II B	17.12 ± 2.66	35	25	23	1	10	28.6
26.6	III	–	33	33	33	–	0	0.0

[a]$P < 0.05$; A = treatment on day 0; B = treatment on day 12

Table 2

Toxicity of Fusilade S to chicken embryos after injection on days 0 and 12 of incubation

Test material and concentration (%)		Body mass (g) of embryo ($\bar{x} \pm$ S.D.)	Number of samples	Embryonic deaths (n)		Embryos showing developmental anomalies (n)	Live embryos	
				total	after treatment		number	%
Control[+]		20.84 ± 1.53	20	6	6	1	14	70.0
0.1	I	22.60 ± 2.53	15	11	11	0	4	26.6
1.0	II A	21.89 ± 2.44	27	13	13	0	14	51.9
10.0	III	10.70 ± –[++]	19	18	18	1	1	5.2
Control[+]		14.96 ± 1.07	15	1	0	0	14	93.3
0.1	I	25.53 ± 3.74[c]	15	4	2	0	11	73.3
1.0	II B	22.82 ± 3.31[c]	26	3	3	0	23	88.5
10.0	III	–	22	22	22	–	0	0.0

[+]Common control group derived from the Maloran 50 WP experiment; [++]based on a single body mass value; [c]$P < 0.001$; A = treatment on day 0; B = treatment on day 12

Table 3

Toxicity of Maloran 50 WP to chicken embryos after injection on days 0 and 12 of incubation

Test material and concentration (%)		Body mass (g) of embryo ($\bar{x} \pm$ S.D.)	Number of samples	Embryonic deaths (n)		Embryos showing developmental anomalies (n)	Live embryos	
				total	after treatment		number	%
Control		20.84 ± 1.53	20	6	6	1	14	70.0
0.1	I	16.81 ± 2.91^{b}	18	9	9	0	9	50.0
1.0	II A	19.70 ± 1.13	26	24	24	0	2	7.7
10.0	III	17.72 ± 3.30^{a}	29	18	18	1	11	37.9
Control		14.96 ± 1.07	15	1	0	0	14	93.3
0.1	I	15.36 ± 1.43	19	1	1	0	18	94.7
1.0	II B	16.25 ± 1.80^{a}	25	3	3	1	22	88.0
10.0	III	18.31 ± 1.68^{c}	30	4	1	2	26	86.7

$^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$; A = treatment on day 0; B = treatment on day 12

Table 4

Combined toxicity of Maloran 50 WP (M) and Flubalex (Fl) to chicken embryos after injection on day 0 of incubation

Test material and concentration (%)		Body mass (g) of embryo ($\bar{x} \pm$ S.D.)	Number of samples	Embryonic deaths, total	Embryos showing developmental anomalies (n)	Live embryos	
						number	%
Control		23.36 ± 2.23	16	4	3	12	75.0
M 1% + Fl 0.266%	I	16.38 ± 3.30[c]	14	0	1	14	100.0
M 1% + Fl 2.66%	II	16.00 ± 1.74[c]	14	1	1	13	92.9
M 1% + Fl 26.6%	III	9.75 ± 1.63[c]	17	13	3	4	23.5

[c]$P < 0.001$

Table 5

Combined toxicity of Maloran 50 WP (M) and Flubalex (Fl) to chicken embryos after injection on day 12 of incubation

Test material and concentration (%)		Body mass (g) of embryo ($\bar{x} \pm$ S.D.)	Number of samples	Embryonic deaths, total/ after treatment	Embryos showing developmental anomalies (n)	Live embryos	
						number	%
Control		25.86 ± 2.91	15	3/2	2	12	80.0
M 1% + Fl 0.266%	I	29.25 ± 1.95	17	3/0	0	14	82.4
M 1% + Fl 2.66%	II	25.30 ± 3.28	18	6/4	3	12	66.7
M 1% + Fl 26.6%	III	–	19	19/17	–	0	0.0

The simultaneous administration of *Maloran 50 WP* and *Fusilade S* on day 0 led to an increase in embryonic mortality in all dose groups. The most expressed increase was found at the two highest dose levels. In the group treated with the highest dose, all embryos died (Table 6). Similar findings were obtained after treatment on day 12, too (Table 7).

The developmental anomalies observed in the different treatment groups occurred sporadically and did not show a dose-response effect (Tables 8–10).

Discussion

In experiments in which the three herbicides were administered separately on day 0 of incubation, Flubalex and Maloran 50 WP typically resulted in a reduction of embryonic body mass, while Maloran 50 WP and Fusilade S led to increased embryonic mortality. The administration of Flubalex was typically followed by an elevated incidence of developmental anomalies in the different groups.

After treatments on day 12, no decrease was found in the body mass of embryos with respect to the control. Post-treatment embryonic mortality was a typical finding in dose group III (10.0%) of Fusilade S and dose groups II and III of Flubalex (2.66% and 26.6%, respectively). A marked rise in the number of developmental anomalies was seen only in dose group I of Flubalex (0.26%).

In tests of combined toxicity, Maloran 50 WP plus Flubalex administered on day 0 of incubation caused a distinct and statistically significant reduction in the body mass of embryos as compared to the control. After the simultaneous use of herbicides this occurred more consistently than when the compounds were used one by one. A significant increase in embryonic mortality was seen only in group III, suggesting that Maloran 50 WP has an expressed effect on embryonic lethality. The number of embryos showing abnormal development did not differ markedly from the control, indicating that in this case the teratogenic effect of Flubalex did not manifest itself.

The simultaneous administration of Maloran 50 WP and Flubalex on day 12 of incubation resulted in expressed embryonic mortality in dose groups II and III. This effect was probably due to Flubalex, as that compound was found to cause embryonic mortality at the two highest dose groups also when administered alone. No decrease occurred in the body mass of embryos as compared to the control. This finding is consistent with the results obtained when the herbicides were administered individually. No difference from the control was observed in the number of developmental anomalies either. This is a favourable finding, as after the administration of Flubalex alone the number of teratological lesions was found to increase in dose group I.

Table 6

Combined toxicity of Maloran 50 WP (M) and Fusilade S (Fu) to chicken embryos after injection on day 0 of incubation

Test material and concentration (%)		Body mass (g) of embryo ($\bar{x} \pm$ S.D.)	Number of samples	Embryonic deaths, total/ after treatment	Embryos showing developmental anomalies (n)	Live embryos	
						number	%
Control		23.36 ± 2.23	16	4	3	12	75.0
M 1% + Fu 0.1%	I	23.13 ± 2.32	16	9	1	7	43.8
M 1% + Fu 1.0%	II	22.73 ± 2.95	17	11	2	6	35.3
M 1% + Fu 10.0%	III	–	16	16	–	0	0.0

Table 7

Combined toxicity of Maloran 50 WP (M) and Fusilade S (Fu) to chicken embryos after injection on day 12 of incubation

Test material and concentration (%)		Body mass (g) of embryo ($\bar{x} \pm$ S.D.)	Number of samples	Embryonic deaths, total/ after treatment	Embryos showing developmental anomalies (n)	Live embryos	
						number	%
Control		25.86 ± 2.91	15	3/2	2	12	80.0
M 1% + Fu 0.1%	I	26.96 ± 3.25	17	6/3	0	11	64.7
M 1% + Fu 1.0%	II	25.63 ± 2.48	17	8/7	0	9	52.9
M 1% + Fu 10.0%	III	–	20	20/17	–	0	0.0

Table 8

Incidence of developmental anomalies in toxicity tests of Maloran 50 WP, Fusilade S and Flubalex in chicken embryos

Groups and days of treatment		Type of anomalies (number of cases)
Control[a]	day 0	Pes debilis (1)
	day 12	–
Maloran 50 WP	day 0	
I		–
II		–
III		Hernia abdominalis et thoracalis, pes debilis, prolapsus cordis (1)
	day 12	
I		–
II		Pes debilis (1)
III		Pes debilis (2), hernia abdominalis, crossed beak (1)
Fusilade S	day 0	
I		–
II		–
III		Pes debilis (1)
	day 12	
I		–
II		–
III		–

a = common control group

After the simultaneous administration of Maloran 50 WP and Fusilade S on day 0, no decrease occurred in the body mass of embryos as compared to the control. This is a favourable observation, since the administration of Maloran 50 WP as a single compound was typically followed by a reduction of body mass. When administered alone, both herbicides caused an increase in the rate of embryonic mortality. This tendency was seen also in the combined toxicity study designed to demonstrate interactions between the two herbicides. The incidence of developmental anomalies was similar in the treated and control groups. The rate of embryonic mortality expressly increased after the individual administration of the two herbicides. That tendency was seen also after their combined use.

The simultaneous administration of Maloran 50 WP and Fusilade S on day 12 of incubation did not cause a reduction in embryonic body mass at any of the

Table 9

Incidence of developmental anomalies in toxicity tests of Maloran 50 WP, Fusilade S and Flubalex in chicken embryos (Table 8 continued)

Groups and days of treatment		Type of anomalies (number of cases)
Control	day 0	Pes debilis (3)
	day 12	Pes debilis (3), anophthalmia sinistra (1), crossed beak (3), subcutaneous cyst on the head (1)
Flubalex	day 0	
I		Pes debilis (4), subcutaneous cyst on the abdominal wall (1), subcutaneous cyst on the head (1) anophthalmia sinistra et dextra, retarded development of mandible (1)
II		Pes debilis (6), subcutaneous cyst on the abdominal wall (2)
III		Pes debilis (7)
	day 12	
I		Pes debilis (8), crossed beak (2), anophthalmia sinistra (2), cerebral hernia (1), subcutaneous cyst on the abdominal wall (1)
II		Pes debilis (1)
III		–

Table 10

Incidence of developmental anomalies in combined toxicity tests of Maloran 50 WP (Ma), Flubalex (Fl) and Fusilade S (Fu) in chicken embryos

Groups and days of treatment			Type of anomalies (number of cases)
Control	day 0		Anophthalmia sinistra (1), crossed beak (1), pes debilis (2)
	day 12		Hernia abdominalis et thoracalis, prolapsus cordis, pes debilis (1)
Ma + Fl	I	day 0	Pes debilis, lordoscoliosis (1)
	II		Anophthalmia sinistra (1)
	III		Pes debilis, oedema generalis, rudimentary beak (1), lordosis, defective ossification of coccygeal vertebrae (1)
Ma + Fl	I	day 12	–
	II		Lordoscoliosis, abdominal cyst, pes debilis (1), lordosis (1)
	III		–
Ma + Fu	I	day 0	Pes debilis (1)
	II		Lordosis (2)
	III		–
Ma + Fu	I	day 12	–
	II		–
	III		–

dose levels used. This finding is consistent with the results obtained after the single use of the compounds studied. At dose level II there was a remarkable rise in embryonic mortality while combined treatment with the highest dose of the test herbicides (dose level III) resulted in expressed embryonic mortality. This was probably due to the presence of Fusilade S, which had been found to have a similar effect also when used alone. The number of embryos with developmental anomalies did not differ markedly from either the control or from the results obtained when the two compounds were used separately.

The gross developmental anomalies observed during the experiments were of sporadic occurrence and were not characterised by dose-response effects or correlations between time of administration and effect. Anomalies of the skeletal system were the most frequent. This is not surprising as the skeletal system has outstanding importance in experimental teratological studies (Tompa et al., 1971). In some cases, developmental anomalies of the soft parts also occurred.

In summary, it can be established that the combinations of two herbicides administered on day 0 in studies of herbicide interaction exerted an adverse effect on the body mass of embryos. The average body mass of the treated groups decreased in the same way as after the separate administration of the two compounds. Treatment on day 12 of incubation had no effect on that variable.

The rate and tendency of embryonic mortality were similar in the combined toxicity studies as after the separate use of the test herbicides, irrespective of the time of treatment. If a given herbicide caused expressed embryonic mortality after use at a specific concentration, it usually maintained that property also in the combined toxicity tests; however, in the latter case no increase of toxicity could be demonstrated.

The incidence of developmental anomalies was not higher after the simultaneous use of herbicide combinations than in teratological tests based on the separate use of the herbicides studied.

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THE EFFECT OF PHENYL MERCURY ON REPRODUCTIVE PERFORMANCE IN LAYING HENS

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The effect of phenyl mercury with and without selenium on the egg production of laying hens and on the fertility, hatchability and properties of eggs was studied. Mercury was administered via the feed at dosages of 5 ppm, 30 ppm, and 30 ppm Hg + 4 ppm Se, for 56 days. After two months, egg production decreased by 8.18% and 7.74% in hens fed 30 ppm Hg, and 30 ppm Hg + 4 ppm Se, respectively. Egg weight decreased in all experimental groups. In comparison to the controls, these results were highly significant ($P < 0.01$) in hens fed 30 ppm Hg and 30 ppm Hg + 4 ppm Se and significant ($P < 0.05$) between hens fed 5 ppm Hg and 30 ppm Hg. Fertility rate and hatchability were not affected. Mercury exposure did not affect egg shape, egg-white height, egg-shell hardness or yolk colour. Both egg-shell thickness and weight decreased in all experimental groups. In the group supplemented with selenium there was a non-significant improvement in egg production, hatchability and all qualitative properties of eggs in comparison with the group without selenium supplementation. Residual mercury levels in egg yolk greatly surpassed the level found in the egg white: the highest values were measured in the group fed 30 ppm Hg. The addition of selenium had a protective effect upon residual Hg deposits in the yolk, but not in the egg-white.

Key words: Phenyl mercury, reproductive performance, laying hens

Mercury (Hg) administered per os shows dissimilar accumulation patterns in different tissues. Inorganic mercury rapidly accumulates in the kidneys, whereas organic Hg slowly accumulates in all organs. The absorption of mercuric salts is 10–15%, and that of alkyl and aryl mercurials is almost complete (95%). Mercury derived from alkyl compounds is readily deposited in eggs (Campabell et al., 1971; Sell, 1977). Most of this type of Hg is found in the egg-white. Szprienger (1975) reported residual Hg levels in egg-white to be twice as high as in the yolk. On the basis of generation experiments carried out in quails, Eskeland et al. (1979) showed the transfer of methylmercury into eggs, the accumulation being higher in the egg-white.

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The protective effect of selenium and its action against Hg was first published by Pařizek and Ošťadalová (1967) and Pařizek et al. (1969, 1971). Sodium selenite in small amounts protected rats against methyl mercury given in the drinking water or in the diet (Ganther et al., 1972; Ganther and Sunde, 1974). This protective effect of dietary Se was also confirmed by El Begearmi et al. (1973), Eybl et al. (1969), Potter and Matrone (1974), Stoewsand et al. (1974), and Mykkänen and Metsänittyl (1987).

The aim of the present study was to determine the accumulation of Hg in eggs after oral ingestion and its influence on the production, fertility, hatchability, and properties of eggs.

Materials and methods

Animal model

Four groups, each containing 12 laying hens (hybrids of the type Shaver Starcross 288 line 589) and 5 cocks (line 579), were used. After the preparation period, the birds were exposed to Hg alone or with sodium selenite (Na_2SeO_2) both added to the feed mixture NVRM. NVRM is a complete feed mixture, with added methionine, for high performance laying hens. Mercury was used in the form of a pesticide containing 5.4% phenyl mercuric chloride over a period of 8 weeks. The dosages were as follows: group 0 = control, group 1 = 5 ppm Hg, group 2 = 30 ppm Hg, group 3 = 30 ppm Hg + 4 ppm Se. Feed and water were supplied *ad libitum*. The mean ration fed to hens and cocks was 120 and 140 g per bird, respectively. The birds were placed in individual cages. Insemination took place at 7-day intervals.

Observations and analyses

Observation of individual indices and sampling were carried out as follows: egg-laying intensity was recorded daily, egg weight was recorded 3 times at 7-day intervals: prior to application, and in the last 7 days of the first and second months after application.

Hardness, thickness, and weight of the egg-shell were examined during the last 7 days of the first and second months of application. Hardness, thickness and weight of the egg-shell were determined according to the method of Marcinka and Gažo (1964). The shape and the internal quality of eggs were observed at the same time intervals as those used for determining the egg weight. The colour of egg yolk was determined according to the paper scale (Hoffman La Roche). Egg shape was determined by measuring the length and width of the egg. Eight batches of eggs were used to follow the hatchability and fertilization of hatching eggs. The eggs

were collected at 7-day intervals. The chickens hatched in the individual groups were reared up to 7 days of age. Six of the hatching eggs were taken from each group at weekly intervals and analysed for the presence of Hg residues both in the yolk and in the white. Mercury levels in eggs were determined in an atomic absorption spectrophotometer.

For light microscopic study, tissue samples from the ovary were fixed in 10% neutral formalin and embedded in paraffin. Histological sections were stained with haematoxylin and eosin.

Statistical analysis

Analysis of variance was used for statistical evaluation of the data. Zootechnical indices were evaluated by Duncan's test.

Results

Egg production

After a 2-month exposure to mercury, egg production decreased by 8.18 and 7.74% in hens fed 30 ppm Hg with and without selenium, respectively (Table 1). In the group fed 5 ppm Hg a slight (3.13%) increase was observed in the egg-laying rate. The results achieved prior to and after the application of Hg are not significantly different.

Egg properties

After 2 months of mercury administration the egg weight decreased in all three experimental groups (98.11, 92.51 and 94.03% in groups 1, 2 and 3, respectively; Table 1). In comparison with the controls, these results were highly significant ($P < 0.01$) in groups 2 and 3 (30 ppm Hg and 30 ppm Hg+Se) and significant differences ($P < 0.05$) were also found between groups 1 and 2 (5 and 30 ppm Hg). In experimental group 3, supplemented with selenium, there was a small improvement in comparison with the group without selenium. Mercury exposure did not affect the parameters of egg shape (Table 2). Numerical data of egg-white height were more favourable in group 3 than in groups 2 and 1 and were even better than in the control group. The colour of the egg yolk was the same in all groups. Both the thickness and weight of the egg-shell slightly decreased in all experimental groups. In contrast to these two characteristics, egg-shell hardness was the best in group 3 supplemented with Se, even better than in the control group.

Table 1

Weight and production of eggs during and after phenyl mercury treatment

Group	On day 28 of the experiment (g)	After the experiment (g)	Comparison with control		Egg production	
			On day 28 of the experiment (%)	After the experiment (%)	Number	%
0 = control	60.20	60.89	100.00	100.00	624	92.85
1 = 5 ppm Hg	60.58	59.74+	102.29	98.11	625	95.98
2 = 30 ppm Hg	55.50	56.33++	92.19	92.51	569	84.67
3 = 30 ppm Hg + 4 ppm Se	56.61	57.26++	94.03	94.03	572	85.11

Asterisks indicate significant differences between the control and the experimental groups: + $P < 0.05$; ++ $P < 0.01$

Table 2

Qualitative properties of eggs after phenyl mercury treatment

Group	Index of egg shape	Colour of yolk	Thickness of egg white (mm)	Hardness of egg-shell	Thickness of egg-shell	Weight of egg-shell
0 = control	1.49	6.50	3.11	8.09	347.80	5.30
1 = 5 ppm Hg	1.54	6.50	3.30	7.98	335.80	5.00
2 = 30 ppm Hg	1.60	6.50	3.40	8.00	335.20	4.90
3 = 30 ppm Hg + 4 ppm Se	1.52	6.50	3.51	8.18	345.50	5.00

Table 3

Fertility and hatchability of eggs after the treatment

Group	Number of eggs in incubation	Unfertilized eggs	Fertility %	Embryos found dead after			Embryos hatched (n)	Embryos hatched (%)
				1st candling	2nd candling	incubation		
0 = control	434	18	95.85	9	5	28	374	86.17
1 = 5 ppm Hg	464	15	96.76	14	9	18	408	87.93
2 = 30 ppm Hg	414	14	96.61	7	5	10	378	91.30
3 = 30 ppm Hg + 4 ppm Se	409	14	96.57	6	1	9	379	92.66

Fertility and hatchability

Fertility was not affected by exposure to Hg during the insemination process. The values obtained in the experimental groups reached a level similar to that of the controls. Hatchability was not affected either; it was even slightly higher in the experimental groups, and the best in group 3 which received Se supplementation. The differences are not significant (Table 3).

Mercury levels

Residual Hg levels in egg yolk and egg-white are given in Figs 1 and 2. In all groups, residual Hg levels in the yolk were several times higher than in the egg white. Mercury levels in the yolk of the group receiving 5 ppm Hg, in comparison to the controls, rose throughout the experimental period, but statistical significance was recorded only during week 4 ($P < 0.01$). Throughout the experiment, statistically significant differences were observed in yolk Hg levels between the groups as follows: control and group 2, control and group 3, groups 1 and 2, groups 1 and 3 ($P < 0.01$). The highest yolk Hg levels were seen in group 2 receiving 30 ppm Hg. The yolk Hg levels of group 3 were lower than those of group 2, but this difference was statistically significant only in weeks 5 and 6 of exposure ($P < 0.05$). On the basis of these results the addition of selenium can be suggested to have a slight protective effect against residual Hg deposition in the egg yolk.

A situation similar to that in the yolk was found in the egg-white, except that no protective effect of selenium was evident. On the contrary, egg-white Hg levels of group 3 were higher, being statistically significant in weeks 1, 4, 5, and 8 ($P < 0.01$) and in weeks 6 and 7 ($P < 0.05$). As to Hg residues in the egg yolk and egg-white, no relationship to the length of exposure was seen.

Histological examination

Histological examination showed a normal process of oocyte development in the experimental groups (Figs 3 and 4). The accumulation on fibrocytes and collagenous fibrils in the ovaries was observed in two hens in experimental group 2. Shrinkage and depressions were observed on secondary and tertiary follicles in one hen.

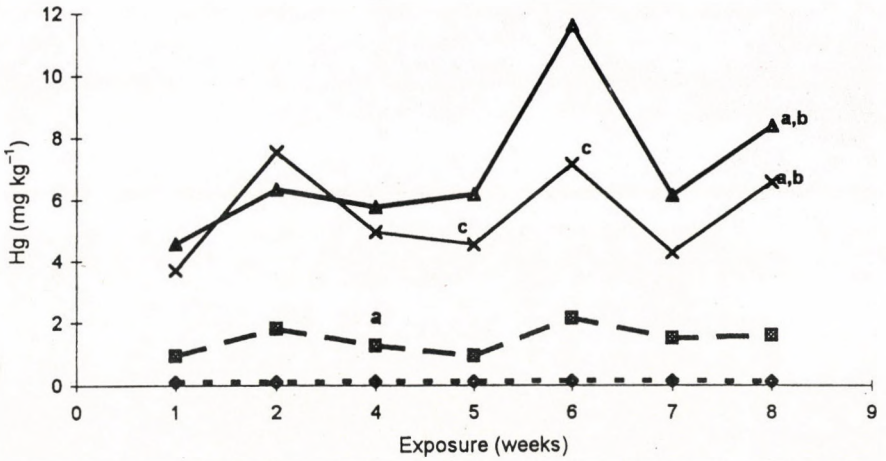


Fig. 1. Levels of Hg in egg yolk after oral exposure to phenyl mercury (5 ppm Hg, 30 ppm Hg or 30 ppm Hg + 4 ppm Se) for 56 days. All values are the mean ± SEM of 6 eggs. The values marked with the letter **a** differ significantly from those of the control group ($P < 0.01$), while values marked with the letter **b** from values of the group fed 5 ppm Hg ($P < 0.01$) throughout the experiment. The values marked with the letter **c** differ significantly from those of the group fed 30 ppm Hg ($P < 0.05$). ◆ - Control; ■ - 5 ppm Hg; Δ - 30 ppm Hg; × - 30 ppm Hg + 4 ppm Se

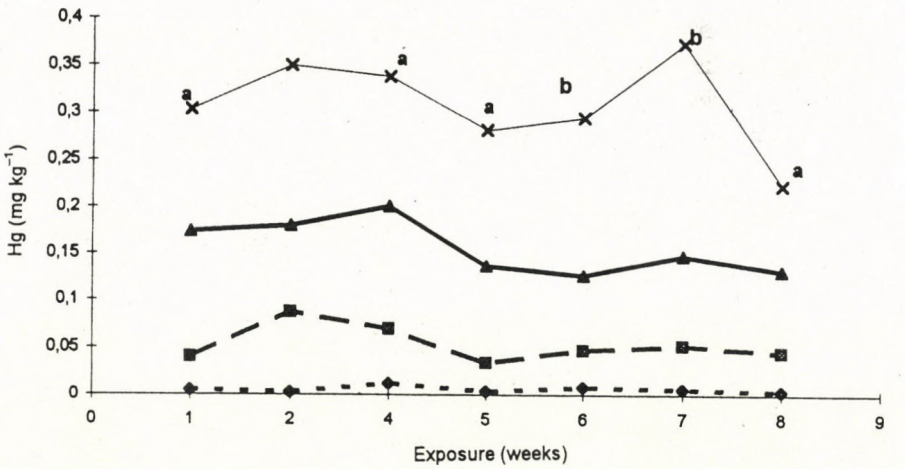


Fig. 2. Levels of Hg in egg-white after oral exposure to phenyl mercury (5 ppm Hg, 30 ppm Hg or 30 ppm Hg + 4 ppm Se) for 56 days. All values are the mean ± SEM of 6 eggs. The values marked with the letters **a** and **b** differ significantly from those of the group fed 30 ppm Hg (**a**: $P < 0.01$, **b**: $P < 0.05$). ◆ - Control; ■ - 5 ppm Hg; Δ - 30 ppm Hg; × - 30 ppm Hg + 4 ppm Se





Fig. 3. Primary follicle and partial section of a tertiary follicle showing normal structure × 150. *Fig. 4.* Secondary follicle of hens treated with 30 ppm Hg shows shrinkage of the granular layer. The oocyte has normal structure. × 150

Discussion

An increased accumulation of mercury in the body causes a deterioration in the breeding properties of fowl, especially in egg production. El Begearmi et al. (1977, 1982) observed that the egg production of Japanese quail administered 15 to 30 ppm Hg in the form of methyl mercury considerably decreased. In this experiment using the same dosage of phenyl mercury no change in egg production was found. Sell (1977) reported a partial reduction in egg production after the administration of 20 ppm Hg as methyl mercury in chickens and quail. A similar effect on egg production was observed also by Maitra and Maitra (1990) after the administration of inorganic Hg. Selenium added at a level of 3 ppm counteracted the effect of 20 ppm Hg to some extent. In the experiment of El Begearmi et al. (1977), the addition of 3 ppm selenium increased the production of eggs by about 11%, whereas in the present experiment the addition of 4 ppm Se was followed by a minimal increase. The administration of mercury in this experiment caused a decrease in the weight of eggs in all experimental groups; the biggest decrease was seen in groups receiving a higher dose of Hg. Scott et al. (1975) reported that feeding 20 ppm Hg as CH_3HgCl to chickens markedly decreased the egg weight and slightly reduced the amount of egg-white; such effects were not observed in the present study. A decrease in egg weight was also observed by Maitra and Maitra (1990) using inorganic Hg. The addition of Se had no significant effect, which is consistent with the results of Scott et al. (1975). The volume of egg-white and yolk and yolk colour did not change considerably but the thickness and weight of the egg-shell decreased in all experimental groups. Egg-shell changes were also observed by Nezel and Vogt (1980) and Maitra and Maitra (1990). The results obtained in this study on the effect of Hg on fertility and hatchability are different from those of other authors. While El Begearmi et al. (1977) who fed 30 ppm Hg to Japanese quail, observed that the hatchability and fertility of eggs decreased to zero, in our experiment these properties did not decrease. On the contrary, fertility and hatchability were even slightly higher in all experimental groups than in the control group after 56 days of experiment. After administering a dosage of 2 ppm Hg acetate for 280 days, Nezel and Vogt (1980) did not observe changes in hatchability. In the present experiment, the addition of Se resulted in a small increase in hatchability, in comparison to the control and the other experimental groups.

There are different views on Hg accumulation in the egg yolk and egg-white. Magat and Sell (1977), Sell (1977) and Eskeland et al. (1979) found a higher accumulation of mercury in egg-white. In contrast to these data, Rissanen and Miettinen (1968) reported the ability of the egg yolk to accumulate higher levels of Hg as compared with the egg-white. Our results also show that the Hg content of egg yolk greatly exceeded that of the egg-white, which corresponds to the

observations of Cibulka and Sova (1986). The reasons for this difference are unknown and the results obtained so far remain contradictory.

It was previously reported that dietary Se increased Hg deposition in the eggs of laying hens. Emerick et al. (1976) reported that eggs of hens fed 5 or 10 ppm Hg (as methyl mercury) and 4 or 8 ppm Se contained significantly more Hg than those of hens fed Hg only. Our results with phenyl mercury and those of Sell (1977) with methyl mercury showed that the concentration of Hg in the yolk was significantly reduced by the administration of Se. In our experiment, deposition of Hg in the egg yolk of chickens fed Hg+Se increased in the second week, whereas in the experiment of Sell (1977) the effect of Se was more consistent, persisting throughout the experiment and disappearing by day 20. According to Sell (1977), the tendency toward an increased accumulation of Hg in the egg-white is higher after the addition of Se. In his experiment, the positive effects of Se on the deposition of Hg in the egg-white of chickens fed 20 ppm Hg was significant by day 4 of the experiment, reaching its maximum on day 8, and then gradually decreasing but still persisting throughout the 21-day trial. A similar effect of Se on the level of Hg in the egg-white was also observed during the first 21 days of our experiment and it persisted throughout the 56 days of the experiment. The slight increase in the Hg level of the egg-white in week 7 of the experiment was somewhat unusual. Like the findings of Sell (1977), our results show that the main effects of dietary Se are to decrease the level of phenyl as well as methyl Hg in the egg yolk and to increase that of Hg in the egg-white. The outcome is a statistically significant interactive effect between dietary Hg and Se on the deposition of Hg in eggs produced by chickens.

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RESTRICTION SITE MAPPING OF A BOVINE ADENOVIRUS TYPE 10 STRAIN

Katalin MATIZ[1,2], Mária BENKŐ[1*], Z. ZÁDORI[1] and B. HARRACH[1]

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Physical maps of the genome of a bovine adenovirus (BAV) type 10 strain (isolate Belfast1) were constructed for eight restriction enzymes. The size of the viral genome was estimated to be around 29,800 base pairs (bp). The orientation of the maps was determined on the basis of partial DNA sequences of the cloned *Pst*I/D fragment of Belfast1. This work is an introduction to DNA sequence and phylogenetic analysis of BAV-10 in order to clarify its taxonomic place.

Key words: Bovine adenovirus type 10, restriction enzymes, physical maps

Adenoviridae is a well-defined virus family spread all over the world (Horwitz, 1990). To date 10 bovine adenovirus (BAV) serotypes have been officially recognised (Russell et al., 1991). BAVs are generally considered to be facultatively pathogenic viruses. The lastly described serotype BAV-10 (Horner et al., 1989), however, seems to play a significant role in enteric disease of cattle (Horner et al., 1980), while the pathogenicity of some other BAV serotypes remains more ambiguous.

The first isolate of BAV-10, strain Ruakura, was isolated in New Zealand from a yearling heifer with haemorrhagic enteritis (Horner et al., 1980). A similar disease was repeatedly observed in Northern Ireland (Smyth et al., 1986), where four successful virus isolations were made from such cases. These four BAV isolates, designated as Belfast1 through 4, were typed as serotype 10 by virus neutralisation tests (Adair et al., 1996). By DNA restriction enzyme analysis, however, the genome of all the four strains seemed to differ more or less from that of the prototype BAV-10 (Benkő et al., 1995) in both size and restriction patterns. Such extensive differences implying the presence of deletions or insertions of considerable size (summing up to 10% of the genome) among the strains of a single BAV serotype have not been reported so far.

BAV-10 has several additional unusual characteristics. Other BAVs could be classified into two subgroups (Bartha, 1969), but BAV-10 seemed not to fit perfectly into either of them (Horner et al., 1989). The poor replication capacity

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restricted to primary cells, the retarded formation of inclusion bodies in bovine testicle cell culture, and the small genome size (estimated to be less than 30 kilobase pairs) are typical of the members of subgroup 2. In DNA hybridisation experiments, however, BAV-10 did not react with the members of either subgroup (Benkő et al., 1995), although the DNA sequence homology had earlier been found to be extensive between the members of subgroup 2 (Benkő et al., 1990).

We have decided to further examine BAV-10 in order to localise the observed deletions/insertions on the genome and to determine their significance. DNA sequencing is also planned in order to obtain data for phylogenetic comparisons and analysis of BAV-10. As a first step, physical maps of the DNA of isolate Belfast1 possessing the largest genome were constructed.

Materials and methods

The Belfast1 strain of BAV-10 was propagated on bovine testicular cell cultures of low passage number. The virions were purified and concentrated by ultracentrifugation, the genomic DNA was phenol extracted as described before (Benkő et al., 1988). The viral DNA was digested with restriction enzymes *Apa*I, *Bam*HI, *Bgl*I, *Cla*I, *Eco*RI, *Kpn*I, *Pst*I, and *Sal*I as specified by the manufacturer (Amersham). The DNA fragments were separated by electrophoresis in agarose gels of different concentrations (0.8–1.6%) depending on the size of the examined fragments. The size of the generated fragments was determined graphically, using λ phage DNA cut with *BstE*II or with *Pst*I enzyme as molecule mass standard. The location and order of the fragments on the BAV-10 genome were determined by overlapping the fragments resulting from single, double or triple digestion. To orient the maps partial DNA sequences (of the two ends, GenBank Accession No. U25262 and U25263) of the previously cloned *Pst*I/D fragment of Belfast1 (Benkő et al., 1995) were used.

Results

The *Apa*I, *Bam*HI, *Bgl*I, *Cla*I, *Eco*RI, *Kpn*I, *Pst*I, and *Sal*I enzymes had, respectively, 3, 7, 3, 5, 3, 2, 5, and 4 recognition sites on the genome of Belfast1. The number and size of the generated fragments are shown in Table 1. The size of the total genome was estimated by adding up the fragments, and the sum was most often found to be 29,800 base pairs (bp).

The size of the fragments generated from double and triple digestion was also estimated (data not shown), and these fragments were overlapped on the fragments resulting from single digestions. The established order of the fragments on the genome is shown in Fig. 1.

Table 1

The size (in base pairs) of DNA fragments generated by restriction endonuclease cleavage of the genome of strain Belfast1

	*Apa*I	*Bam*HI	*Bgl*I	*Cla*I	*Eco*RI	*Kpn*I	*Pst*I	*Sal*I
A	15,600	12,230	21,300	11,200	14,000	21,200	15,650	8,300
B	10,300	6,870	4,300	8,650	13,600	8,100	6,650	7,900
C	3,000	4,500	3,500	5,300	1,600	500	2,250	7,900
D	900	3,700	700	2,400	600		2,100	3,500
E		1,300		1,450			1,900	2,200
F		950		800			1,250	
G		125						
H		125						
Total	29,800	29,800	29,800	29,800	29,800	29,800	29,800	29,800

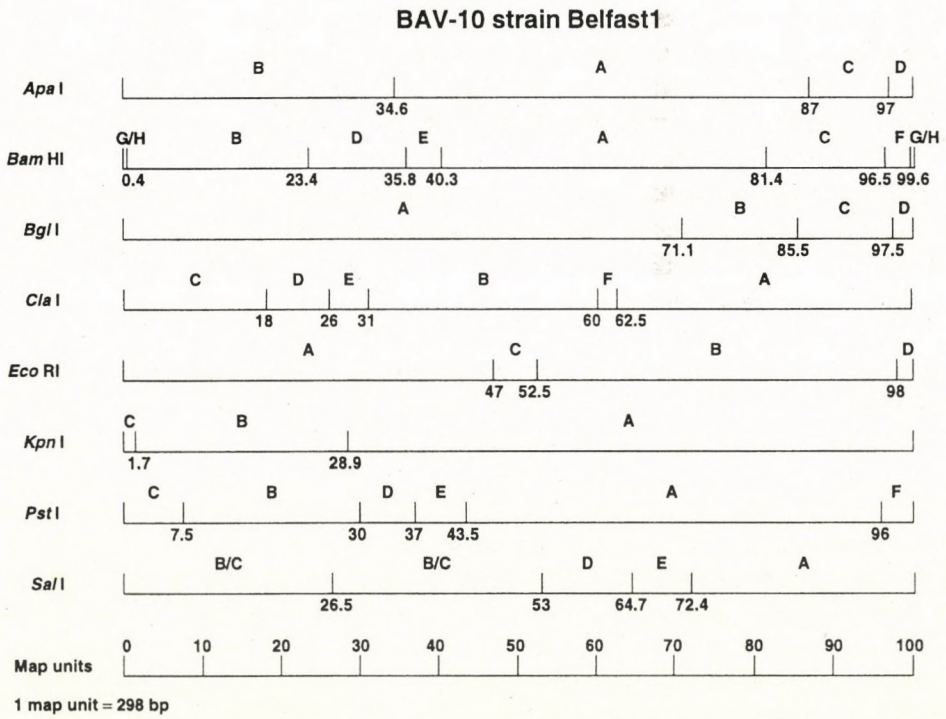


Fig. 1. Physical maps of Belfast1. Location of the restriction enzyme cleavage sites is given in map units. [This figure is available through Internet (http://www.vmri.hu/harrach/index.htm)]

Computer analysis of the earlier determined DNA sequence and deduced amino-acid sequence of the two ends of the *Pst*I/D fragment of Belfast1 allowed us to identify the genes coding for the 52K and the IIIa proteins. The location of these genes in the genome of human adenovirus type 2 (HAV-2) is between map units 30 and 40 (Horwitz, 1990). The restriction site maps (presented in Fig. 1) were oriented supposing that the genome organisation of BAV-10 is similar to that of HAV-2 and thus the *Pst*I/D fragment should be placed on the left hand part of the map.

Discussion

There are at least three good reasons why BAV-10 is an interesting bovine adenovirus and deserves further study. Firstly, BAV-10 is presently represented by five isolates, all of which had been isolated from fatal haemorrhagic and/or fibrinous enteritis cases in cattle. Although its pathogenic capacity has not been confirmed by experimental infection, BAV-10 seems to be more clearly related to disease than are other BAV serotypes which have repeatedly been isolated from primary bovine tissue cultures or clinically healthy animals as well. At the same time, the BAV-10 strains did not cross react with subgroup 1 or subgroup 2 BAVs in DNA hybridisation experiments (Benkő et al., 1995), therefore a BAV-10-type-specific diagnostic method (*in situ* DNA hybridisation, Smyth et al., 1996) could be elaborated. Retrospective examination of earlier cases of similar pathology revealed the occurrence of BAV-10 in several additional European and American countries (Smyth, personal communication). The veterinary importance of BAV-10 might therefore be somewhat more remarkable than is thought to be now.

Secondly, the calculated genome size (29,800 bp) of Belfast1 is relatively small, but the other BAV-10 isolates seem to have an even shorter DNA (Benkő et al., 1995). The presence of possible deletions (or insertions) accounting for up to 10% of the genome is an interesting finding. Since all the five BAV-10 isolates originated from fatal cases, the observed genetic variation of the BAV-10 strains might also be related to pathogenicity. On the basis of the physical maps presented here and the restriction enzyme patterns of the BAV-10 isolates (Benkő et al., 1995) the variations could be defined between map units 72.4 and 96.5. Supposed that the genome organisation of BAV-10 is comparable to that of other mammalian adenoviruses, this region may involve the E3 transcription unit (Horwitz, 1990). In most human adenoviruses, E3 is a fully dispensable genome region encoding for proteins that interact with the host's immune system (Wold et al., 1995).

Lastly, since BAV-10 does not fit into either BAV subgroup, there is a chance that this virus is not a genuine bovine virus but perhaps originated from another host species, and is now in the progress of adaptation to cattle. Although adenoviruses are generally considered as strictly host species specific microorganisms, the isolation of BAVs from sheep has been reported in several instances (Belák and Pálfi, 1974; Adair et al., 1985). Egg Drop Syndrome virus is also known to be capable of infecting more than one bird species (McFerran et al., 1978). Genomic variations of such adenovirus isolates from different hosts have been noted (Belák et al., 1986; Todd et al., 1988; Boyle et al., 1994). The genome variation of BAV-10 could well be the sign or result of genome rearrangement due to adaptation. Nonetheless, if the natural host of BAV-10 is cattle, based on its genetic characteristics it might be considered a representative of a new (third!) subgroup of BAVs.

Our further plans include the construction of physical maps of the other four BAV-10 strains. This work is facilitated by the present results. Molecular cloning and DNA sequencing of the Belfast isolates is now in progress with the purpose of defining the genetic identity of the variable (deleted?) regions, as well as of gaining sequence data for phylogenetic comparisons.

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MAGYAR
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BOOK REVIEW

Eurovet Guide/A Guide to Veterinary Europe 1996–1997. Les Éditions du Point Vétérinaire, France, 1996. 1st edition. Editor: Karin de Lange, DVM, MRCVS. 480 pages. Price: 295 FF.

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BODY COMPOSITION OF MICE OF DIFFERENT BODY CONDITION SCORE AND SEX

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After a critical review of recent research on the whole body chemical composition of laboratory animals, especially that of the mouse, the authors present data on the body composition of a mouse line of different body condition score, varying from normal to double-muscled ("culard", "compact") type. One hundred male and 100 female mice of different compact categories were measured *in vivo*, using total body electrical conductivity (TOBEC method), then by direct chemical analysis performed on day 77 of life. In male mice, body weight and protein content were found to increase while fat content decreased parallel to the increase in phenotypic condition score. Female mice showed a rather constant fat, protein and ash content in the dry matter of the body. The precision and validity of TOBEC data under 40 grams of body weight proved to be insufficient.

Key words: Mouse, body condition score, body composition, chemical analysis, total body electrical conductivity

Studies on whole body composition have a long history (Von Bezold, 1857; Moulton, 1923; Spray and Widdowson, 1950). Owing to the genetic, endocrinological and biotechnological advances made in recent years, however, the topic now commands renewed interest. As a result of the use of these techniques in animal husbandry the body composition of animals may change; therefore, the study of these modifications is essential both from the theoretical and the practical point of view. Several methods have been developed for measuring whole body composition (Fekete, 1991). The common principle can be postulated after Bertrand Russel (Rhys, 1932) as follows: "Although this may seem a paradox, all exact science is dominated by the idea of approximation." Consequently, in case of doubt, or when introducing a new method, the basis of calibration and validation should be the direct chemical analysis.

The practical aspect of studies of this type is that animal production is making strong efforts to reduce carcass fat in response to consumer demands. Methods of minimising fat content include nutrition, application of repartitioning agents, immunisation against specific hormones, and genetic selection. Laboratory

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animals, especially the mouse, have proven to be a useful model for studying the factors influencing growth rate and body composition (Eisen, 1989).

In this short review of the relevant literature we concentrated on those studies in which the body composition of a conventional (normal) laboratory mouse had been compared to that of a modified one. Selection for rapid growth rate is usually accompanied by a positive correlated response in per cent body fat. The polygenic obese strain of mice provides a good model for studies on the aetiology of obesity. Padua-Deodato et al. (1982) fed polygenic obese, non-obese and reciprocal crossbred mice with a purified diet containing 1, 5 or 25% corn oil from 3 to 10 weeks of age. Epididymal and subcutaneous fat depot weight and proportion were used as measures of adiposity. At 10 weeks of age, most of the line differences in fat depots was due to additive direct genetic effects, while the contribution of maternal genetic effect was negligible. Epididymal and subcutaneous fat significantly increased with the dietary fat per cent, but subcutaneous fat was not affected. Interactions of genotype with the level of dietary fat were not significant. Eisen and Coffey (1990) also stated that although there are high positive correlations between fat depots in mice (and chickens), local control of lipogenesis and/or lipolysis exists at different sites of fat deposition. Consequently, the knowledge of total body composition is indispensable.

Investigations into the effects of growth hormone (GH) on carcass composition have consistently shown a reduction in the proportion of fat in the carcass. Bootland et al. (1991) studied the effects of exogenous GH on growth and body composition of mice selected for high or low body weight, or high or low body fat. The animals were given daily injections of recombinant bovine GH (9 μg/g body weight). Treatment with GH increased the rate of weight gain and final weight in mice of all lines. Tibia length was consistently greater in treated animals. In both lines selected for body fat, GH decreased fat content. The mice of low body weight also became less fat, but in mice of high body weight the application of GH increased fat percentage. According to Holder et al. (1988), the action of bovine growth hormone might be enhanced by simultaneous treatment with a special monoclonal antibody in hypopituitary dwarf mice: increased body weight, length, less body fat and more protein could be measured.

The effect of a potential feed additive, the beta-2-agonist clenbuterol (3 mg/kg diet) with or without growth hormone (15 μg/growing dwarf mouse) was studied by Bates and Pell (1991). Clenbuterol alone did not affect body weight or length. GH, with or without clenbuterol, induced an increase in body weight and tail length growth. Both treatments reduced the percentage of whole body fat and increased the protein:fat ratio.

Bradford (1982) identified the autosomal major gene (high growth, "*hg* gene") of the rapid postweaning growth of mouse. This gene is expressed as a ho-

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mozygous recessive, which increases postweaning growth by 60% in C57Bl/6 mice, compared to the same genetic stock without the major gene. Calvert et al. (1984) studied the body composition of normal (*Hg*) and *hg* mice at 49 days of age. The liveweight of *hg* animals was significantly higher (45.6 vs. 34 grams). Percentage of body water (66.75%) was not affected by genotype. When calculated to the dry matter, only the protein content was significantly affected by genotype, being higher for *hg* males. It was also stated that the differences were the result of a slower rate of maturation, and that the *hg* gene had only a general influence on all chemical components of growth and did not alter any of its specific chemical components (Calvert et al., 1985). Absolute protein synthesis was 40% greater in line *hg* than in normal mice, measured at days 21, 31 and 42 of age (555, 1224 and 1216 vs. 680, 1720 and 2222 mg/day). The relative contributions of liver, gastrointestinal tract, heart-kidney-lung and remaining carcass to whole body protein absolute synthesis rates were not affected by line, but did change with age (Bernier and Calvert, 1987). Bernier et al. (1986) examined the effect of the *hg* gene on the maintenance energy requirement and net energetic efficiency of mice between days 21 and 42 of age in a comparative slaughter trial. Animals were fed *ad libitum* with a purified diet. Mice with the *hg* gene line gained 46% more total body energy per unit of metabolic body weight ($kg^{0.75}$), while their energy intake was equal. The increased energy gain was largely (78%) due to an increase in fat energy deposition.

The cited works suggest a need for simple and non-invasive techniques for measuring body composition even *in vivo*. One of those is the device that estimates lean tissue by electromagnetic means. Lean tissue is a much better conductor of electricity than is fat. The instrument is basically a long coil driven by oscillating radiofrequency current. The actual measurement consists of the difference between the coil impedance when empty and that when a sample (the whole organism) is inserted (Harrison and Van Italie, 1982; Presta et al., 1983).

The goal of the present investigation was to elucidate the relationships between the phenotypic condition score and the body composition of the compact mouse line, and to study the influence of sex and the applicability of total body electrical conductivity measurement.

Materials and methods

One hundred male and 100 female mice of the compact line (Varga et al., 1993), ten per each phenotypic condition score, were included in this investigation.

In this body condition scoring system, in which measuring is done by watching and touching the animals, body condition score 1 (BCS 1) represents the normal phenotype while BCS 5 corresponds to the heavily muscled type of animal.

After weaning on day 21, the mice were kept under controlled temperature (20 °C) and light conditions (14 h light:10 h darkness). They were given free access to tap water and to a pelleted mouse diet (LATI diet number 2, LATI, Gödöllő, Hungary). The nutrient concentrations of the natural ingredients (cereals–soybean) corresponded to the NRC (1995) allowances, containing 16.2 MJ/kg ME, 18% crude protein and 5% fat.

On days 70–77 of age the animals were weighed and their condition score was estimated. Whole body electrical conductivity was measured in ketamine + xylazine narcosis, using an EM-SCAN® Model-SA-2 equipment (Springfield, IL, U.S.A.). This machine works on the above-mentioned electromagnetic principle (Presta et al., 1983) and measures total body electrical conductivity ("E" number). By direct computer interfacing it displays total lean body mass, per cent body fat and total body water. On each individual 10 measurements were done and the mean was used in subsequent studies.

Subsequently, the mice were put to sleep by an intraperitoneal injection of pentobarbital sodium, deep frozen, ground and the water/dry matter, ash, crude protein (N × 6.25) and ether extract content determined according to the A.O.A.C. (1975) recommendations. For further details of the methodology, see Fekete and Brown (1993).

Statistical analyses (*t*-comparisons, calculation of linear correlation coefficients) were carried out after Pearce (1965), using the EXCEL 4 software (Barakonyi, 1993).

This study was approved by the Animal Use and Care Administrative Advisory Committee of the Hungarian Scientific Chamber, and it complies with European Union directives regarding the use of experimental animals.

Results

Since sex generally has a great influence on body composition (Fekete, 1992), the data are pooled by sex and body condition score.

Table 1 shows the body weight, chemical composition (dry matter, ash, crude protein, ether extract = fat concentration) and "E" value of male while Table 2 presents those of female mice, grouped according to the BCS.

Figures 1 and 2 illustrate the composition of dry matter according to the phenotypic body condition scores.

Table 3 summarises the correlation coefficients between the BCS, body weight, major chemical components and total body electrical conductivity number (E value).

Table 1

Composition of the whole body: males (mean ± standard deviation)

BCS	LW (g)	DM (%)	Ash (%)	CP (%)	Fat (%)	E-value
1	26.93 ± 2.56	33.40 ± 1.33	3.26 ± 0.26	18.44 ± 0.83	9.96 ± 1.69	10.23 ± 1.48
2	29.71 ± 2.90	32.58 ± 2.65	3.07 ± 0.20	18.06 ± 0.71	9.42 ± 3.02	12.13 ± 1.66
3	34.40 ± 3.03	31.61 ± 2.16	3.21 ± 0.19	18.82 ± 0.66	8.18 ± 2.68	13.06 ± 1.95
4	34.76 ± 3.88	31.23 ± 2.61	3.01 ± 0.19	18.70 ± 0.69	7.66 ± 3.02	12.29 ± 1.77
5	36.25 ± 3.88	29.50 ± 1.31	2.98 ± 0.22	18.79 ± 0.56	6.09 ± 1.44	11.06 ± 1.09

BCS = body condition score; LW = liveweight; DM = dry matter; CP = crude protein; EE = ether extract (fat); E-value = total body electrical conductivity number

Table 2

Composition of the whole body: females (mean ± standard deviation)

BCS	LW (g)	DM (%)	Ash (%)	CP (%)	Fat (%)	E-value
1	27.84 ± 2.16	30.70 ± 1.70	3.53 ± 0.29	17.56 ± 0.48	9.15 ± 1.95	12.20 ± 1.65
2	27.88 ± 2.81	32.64 ± 2.49	3.29 ± 0.16	17.66 ± 0.81	10.13 ± 2.49	11.08 ± 1.87
3	28.40 ± 2.01	31.45 ± 3.44	3.27 ± 0.19	17.64 ± 0.63	8.98 ± 3.59	10.80 ± 3.33
4	29.10 ± 1.79	33.34 ± 2.12	3.21 ± 0.15	17.12 ± 0.60	10.51 ± 2.37	11.40 ± 2.03
5	30.66 ± 1.95	30.09 ± 2.68	3.11 ± 0.17	17.18 ± 0.60	9.28 ± 2.38	12.10 ± 1.57

BCS = body condition score; LW = liveweight; DM = dry matter; CP = crude protein; EE = ether extract (fat); E-value = total body electrical conductivity number

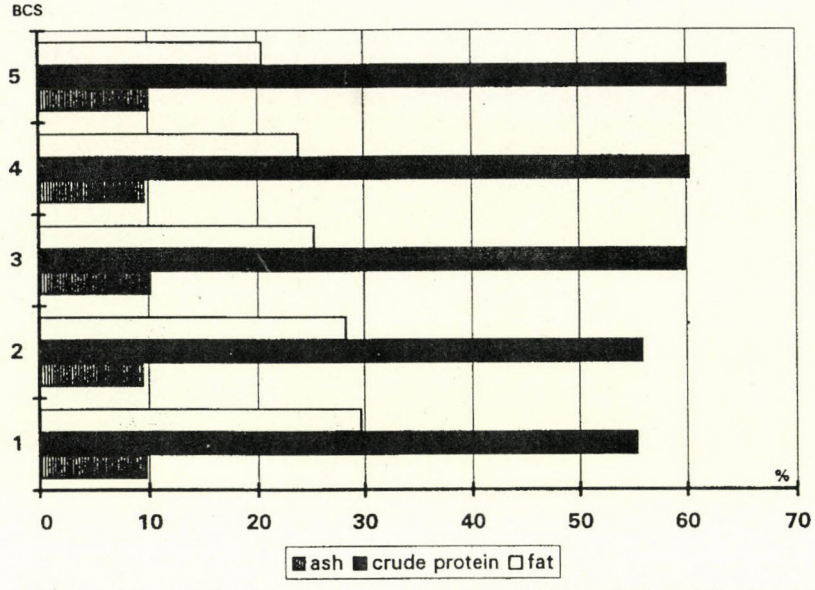


Fig. 1. Composition of the dry matter in male mice of different (1 to 5) body condition scores: fat (striated columns), protein (dotted columns), ash (black columns)

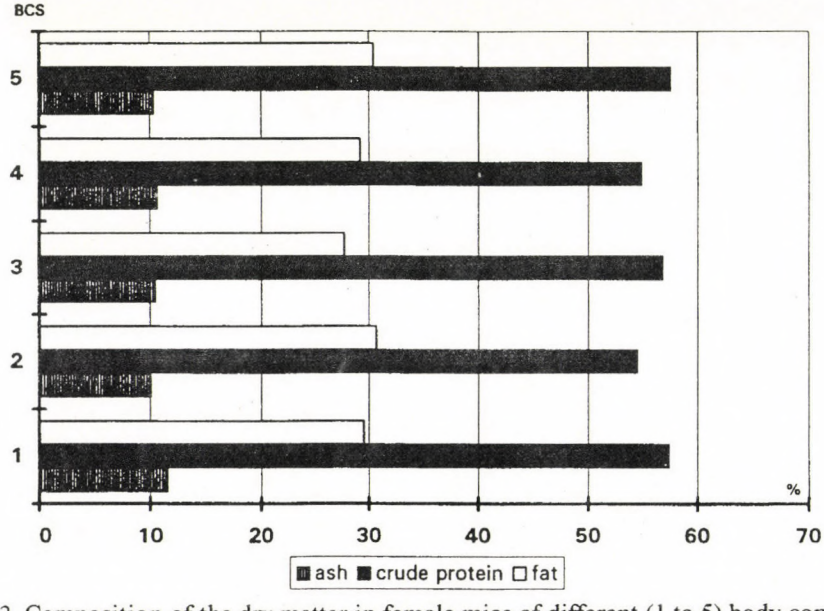


Fig. 2. Composition of the dry matter in female mice of different (1 to 5) body condition scores: fat (striated columns), protein (dotted columns), ash (black columns)

Table 3

Correlation coefficients among the major chemical components and the E-value

Parameters	Body weight		Dry matter		Ash		Protein		Fat	
	M	F	M	F	M	F	M	F	M	F
In the whole body										
Dry matter	–0.17	0.02	1.00	1.00	xxx	xxx	xxx	xxx	xxx	xxx
Ash	–0.05	–0.44*	–0.05	–0.04	1.00	1.00	xxx	xxx	xxx	xxx
Protein	0.27*	–0.32	–0.59*	–0.51*	0.40*	0.35	1.00	1.00	xxx	xxx
Fat	–0.13	0.08	0.97*	0.92*	–0.17	–0.13	–0.72*	–0.71*	1.00	1.00
E-value	0.43*	0.94*	0.24*	0.01	0.17	0.36	0.02	–0.03	0.22	–0.07
In the dry matter										
Ash	0.01	–0.31	xxx	xxx	1.00	1.00	xxx	xxx	xxx	xxx
Protein	0.24*	–0.12	xxx	xxx	0.79*	0.78*	1.00	1.00	xxx	xxx
Fat	–0.14	0.11	xxx	xxx	–0.80*	–0.70*	–0.98*	–0.92*	1.00	1.00
E-value	0.43*	0.94*	xxx	xxx	–0.04	–0.24	0.16	–0.10	xxx	xxx
In the fat-free dry matter										
Ash	–0.03	–0.33	xxx	xxx	1.00	1.00	xxx	xxx	xxx	xxx
Protein	0.53*	–0.09	xxx	xxx	0.02	0.44*	1.00	1.00	xxx	xxx
E-value	0.43*	0.94*	xxx	xxx	0.22*	–0.26	0.09	–0.08	xxx	xxx

*Correlation coefficient significantly ($p < 0.05$) differs from zero; M = male, F = female

Discussion

The experiment was finished when the animals were 70–77 days old. That time was chosen for two reasons: by that age mice can be considered sexually mature (Havenaar et al., 1993) and the body condition score is well recognisable. The liveweight of female mice practically did not differ among the different BCS groups (30.66 ± 1.96 g), while that of males showed a consistent but non-significant increase parallel to the BCS (26.93, 29.71, 34.40, 34.76 and 35.25 g, respectively). The average dry matter content of the body did not differ significantly when female and male mice were compared (30.09 vs. 31.66%), but in the latter there was a consistent decrease parallel to the increase in BCS (33.40, 32.58, 31.61, 31.23 and 29.50%, respectively).

Analysing the composition of the dry matter on the basis of Figs 1 and 2, it can be stated that the data of females show no regularity according to the BCS. On the contrary, the data of males show a clear regularity: parallel to the increase of BCS, the crude protein content elevates and the fat content decreases. The ash remains practically the same. In other words: the more muscled the mouse, the higher the protein and the lower the fat concentration of its body dry matter.

The most interesting correlations are shown in Table 3. In males, the liveweight has a positive correlation with protein content in the whole body ($r = 0.27$), in the dry matter ($r = 0.24$) and in the fat-free dry matter ($r = 0.53$). No correlation can be found in the case of females. Both sexes show a good correlation ($r = 0.43$ and 0.94) between the liveweight and the E value. The protein concentration, except that of the fat-free dry matter of males, has a close positive correlation with the ash content. The correlation of fat and dry matter is high in both sexes ($r = 0.97$ and $r = 0.92$ in males and females, respectively). Between the fat and protein content of dry matter there is a very close negative correlation ($r = -0.98$ and -0.92), which justifies extrapolation from one parameter to the other in the mouse like in other species (Fekete, 1991).

The E value (which reflects the total body electrical conductivity) shows a good correlation only with the liveweight in both sexes (0.43) and with dry matter in males (0.24); however, it often showed illogical data (e.g. $r = -0.36$) with the total body ash in females. To tell the truth, the equipment supplier made a slight remark in his material in brackets: "Repeatability may be affected below 40 g". Based on these results, one can state that in the case of mice of low liveweight (20–30 grams) this method cannot be used for obtaining reliable individual data.

To compare our data with the relevant international literature, Table 4 presents a summary. It can be stated that there are big liveweight differences among different breeds. Data obtained in different trials on all major chemical components but fat are relatively similar. The protein values obtained in the present trial

tend to be lower while those of fat higher. This can be clearly demonstrated by the lower protein:fat ratio. The data suggest that the mouse line used is an obese rather than a lean one.

Analysing the data obtained for the two sexes in the present trial, it can be established that the body of males contains more protein and less fat than that of females of the same BCS. This can be well demonstrated when expressing the protein:fat index of males as a percentage of that of females of the same condition score: BCS 1 = 0.96%, BCS 2 = 113%, BCS 3 = 124%, BCS 4 = 152% and BCS 5 = 164%. Shebaita (1977) established important sex differences in the mouse. The higher fat content of females might be related to the finding of Frisch (1994), who stated that the onset of cyclic sexual activity requires a certain proportion of body fat.

Comparing the two sexes in terms of ash content (Tables 1, 2 and 4), the females show a consistently but non-significantly higher value. The data suggest differences in the stage of chemical maturity of the two sexes. Moulton (1923) found that mammals become chemically mature at different ages, the proportion of the total life span that elapsed prior to the attainment of chemical maturity was approximately 4% for all species investigated: 50 days in rats, 50 days in guinea-pigs, 100 days in cats, 200 days in dogs, 150–300 days in pigs, 150–300 days in cattle, and 500–1000 days in humans. Chemical maturity means the age at which the concentration of water, protein and ash in the fat-free body becomes constant. Chemical maturity is closely associated with the age at sexual maturity (Malik, 1984).The usual life span of the mouse is about 30 months (Silberman and Kedar, 1977). On the basis of the above-mentioned data, the mouse attains chemical maturity at the age of 5–7 weeks. To elucidate the phenomenon, we calculated some parameters of the fat-free dry matter (ash 14.93 vs. 13.27 and protein 82.62 vs. 79.31% in females vs. males, respectively). As an indicator of chemical maturity, the ratio of protein:ash has been determined for the different body condition scores (female–male order): BCS 1 = 4.97 vs. 5.67, BCS 2 = 5.38 vs. 5.86, BCS 3 = 5.33 vs. 5.86, BCS 4 = 5.32 vs. 6.20, and BCS 5 = 5.54 vs. 6.32. These numbers suggest that males of this mouse line have not yet reached the stage of chemical maturity. Further investigations are needed, parallel to gene mapping, to determine the exact time of puberty, breeding, and chemical maturity of both sexes.

Table 4

Body composition of mice: a comparison of previous studies and the present trial

Author	Breed	Age (days)	Sex	Weight (g)	Water (%)	Protein (% of DM)	Fat (% of DM)	Ash	Protein to fat ratio
Bates and Pell (1991)	dwarf	71	M, F	8.32	–	10.41*	25.4*	–	0.41
Holder et al. (1988)	dwarf	63–70	M, F	8–11	56.9*	14.10*	17.4*	–	0.80
Bootland et al. (1991)	fat	48	M	29.7	61.4*	2.66x**	–	–	–
	lean	48	M	27.0	68.2*	x**	–	–	–
	rapid growth	48	M	38.1	66.9*	1.15x**	–	–	–
	slow growth	48	M	23.6	64.0*	2.32x**	–	–	–
Padua-Deodato (1982)	obese	70	M	57.0	–	–	–	–	–
	normal	70	M	37.0	–	–	–	–	–
Calvert et al. (1984)	normal	49	F	34.0	–	–	–	–	–
		49	M	45.6	67.8	64.6	20.8	11.5	3.10
	hg	49	F	27.3	–	–	–	–	–
		49	M	33.2	66.7	60.7	26.1	10.2	2.33
Calvert et al. (1985)	normal	57	M	31.9	69.5	68.2	15.7	11.8	4.34
	hg	57	M	44.2	70.1	68.6	17.1	10.7	4.01
Present trial (1996)	BCS 1	70–77	F	27.8	69.3	57.4	29.6	11.5	1.94
		70–77	M	26.9	66.6	55.3	29.7	9.8	1.86
	BCS 2	70–77	F	27.9	67.4	54.1	31.0	10.0	1.75
		70–77	M	29.7	67.4	55.9	28.4	9.5	1.97
	BCS 3	70–77	F	28.4	68.5	56.1	29.5	10.4	1.90
		70–77	M	34.4	68.4	59.9	25.5	10.2	2.35
	BCS 4	70–77	F	29.1	66.7	51.6	31.2	9.7	1.65
		70–77	M	34.8	68.7	60.4	24.0	9.7	2.51
	BCS 5	70–77	F	30.7	69.9	57.5	30.5	10.4	1.89
		70–77	M	35.3	70.5	63.8	20.5	10.0	3.11

M = male; F = female; "–" = no data available; *% of whole body; **measured by the gonadal pad fat

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EVALUATION OF THE DIGESTIBILITY OF NUTRIENTS IN PIGS BY ILEAL CANNULATION TECHNIQUE

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A simple T-cannula was surgically inserted into the ileum of growing female Large White × Dutch Landrace swine. Chymus samples collected with the help of the cannula were analysed to determine the apparent digestibility of different dietary nutrients such as dry matter, crude protein and starch, as well as of some essential amino acids. The following three experiments were conducted: (I) of the cereals, the "Tewo" triticale variety fed alone and in 1:1 concentrate mixtures with wheat or maize was studied; (II) waxy maize hybrids and maize hybrids of normal endosperm were compared without treatment and after treatment by the Bocchi technology; (III) untreated and extruded maize supplemented either with extracted soybean or with extracted sunflower was also tested. The ileal digestibility of protein, amino acids and starch was determined and compared with values obtained by conventional (faecal) analysis. When feeding triticale alone or in combination with wheat or maize, the ileal digestibility of crude protein and amino acids increased as a result of feeding the cereal combinations (e.g. crude protein 72–79%, lysine 75–79%, threonine 63–78%, methionine 74–86%). Comparison of the two maize hybrids revealed that, with the exception of methionine, lysine and tyrosine, the amino acids of the waxy hybrid had higher ileal digestibility. Treatment of the normal hybrid by the Bocchi technology caused a significant improvement in the ileal digestibility of cystine, isoleucine, lysine, valine, and dry matter. This treatment also improved the faecal digestibility of all test nutrients but methionine. Bocchi treatment of the waxy hybrid significantly improved the ileal digestibility of isoleucine, leucine, methionine, tyrosine and valine, and the faecal digestibility of cystine, dry matter, and crude protein. No major variety- or treatment-related differences were found in the digestibility of starch. As a result of extrusion, the digestibility of nutrients of the soybean + maize mixture increased from 61.6% to 70.3% (crude protein), from 41.1% to 59.4% (threonine), from 60.1 to 72.0% (methionine), and from 70.7% to 82.7% (lysine). The same treatment of the sunflower + maize mixture increased the digestibility of crude protein from 80.6% to 84.5%, that of threonine from 78.1% to 80.6%, that of methionine from 79.7% to 84.3%, while that of lysine from 61.4% to 72.3%. The ileal digestibility of starch was 97–98% for both mixtures. As a result of extrusion, most of the faecal digestibility values showed a significant improvement for both the soybean- and the sunflower-containing

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mixtures. The favourable effect exerted by extrusion on the digestibility of nutrients is markedly influenced by the feed components.

Key words: Pig, small intestine, T-cannula, feed treatments, protein, amino acid, starch, ileal digestibility

The nutritive value of feeds is determined by nutrient content and digestibility. Digestibility is usually measured by the classical method of faecal analysis, when the results of chemical analysis of the feed are compared with the corresponding values obtained by faecal analysis, and the digestibility of the diet is determined on the basis of the difference. However, from what we know about digestive processes it is obvious that this method has certain inherent shortcomings. In the proximal part of the digestive tract plenty of digestive secretions get into the gut, part of which will be decomposed and absorbed. The chemical composition of the intestinal content markedly changes as a result of fermentation processes and bacterial activity taking place in the caecum and the large intestine. These changes are not taken into consideration during the faecal analysis. Thus, the digestibility value measured by faecal analysis does not apply to the diet alone: it is modified by the effects of enzymes, bacteria and their products participating in the digestion of the diet.

More accurate digestibility coefficients of dietary nutrients can be obtained by the determination of ileal digestibility. Ileal digestibility coefficients are usually lower than faecal ones, but more closely reflect the degradation of the diet studied and the absorption of its useful ingredients.

Ileal digestibility values can be used successfully for the qualification of basic feedstuffs and their mixtures. In addition, they are suitable for determining the effects of technical feed processing steps such as heat treatment and increase of particle surface (flaking), or the success of inactivation of antinutritive substances (e.g. trypsin inhibitor). In order to support these statements, the following three experiments were carried out, using growing pigs provided with an ileal cannula: (I) Determination of the digestibility of nutrients of a newly improved "Tewo" triticale when fed alone or in 1:1 mixtures with wheat or maize. (II) Comparison of waxy hybrid maize and a hybrid maize of normal endosperm without treatment and after treatment by the Bocchi technology. (III) Study of untreated and extruded feed mixtures. The objective of the experiments was to determine the digestibility of crude protein, certain amino acids and starch when using the above mixtures or technical treatments. In addition, the obtained values were compared with the results of conventional faecal analysis.

Materials and methods

Large White × Dutch Landrace F_1 gilts weighing 30 ± 2 kg were used. A simple T-cannula was surgically inserted into the pigs' ileum, at a distance of about 15 cm from the caecum. The surgical intervention and the postoperative treatment and keeping of pigs were done as described by Kubovics et al. (1989).

Four pigs fitted up with an ileal cannula were used in each experimental treatment. The daily ration of the pigs corresponded to 3.5% of their body weight. During the 9-day prefeeding period and in the 5-day sample collection phase the pigs consumed that ration in the form of a 1:1 aqueous solution at two meals given at 7:00 a.m. and 15:00 p.m. The feed was supplemented with 8 g Cr (III) chromic oxide (in the form of Cr-NDF) as particle marker.

The schedule of feeding and collection of chymus and faecal samples is shown in Fig. 1. The 14-day trial consisted of a 9-day prefeeding period and a 5-day sample collection phase. The feeding of marker (Cr-NDF) was started 5 days before the start of the sample collection phase. Chymus was collected on three days of the 5-day sample collection phase, four times daily, at specific intervals, over a period of 80 minutes.

Chymus was collected into a plastics bag (approx. 1 g, 5 × 25 cm) screwed on the cannula with the help of a threaded ring. When the bag was filled with chymus up to two-thirds, it was removed, sealed airtight, and immediately placed into a refrigerator. At the end of each collection day, the chymus samples collected from individual animals were put away for storage at −18 °C until used for laboratory analyses.

Faecal samples were collected at the time of chymus collection separately from individual animals in a representative manner, and were stored deep frozen at −18 °C until used for the chemical assays.

Experimental diets

In experiment I, "Tewo" triticale as well as its mixtures with either wheat or maize were studied. The composition and nutrient content of the feed mixtures are shown in Table 1.

In experiment II, waxy maize hybrids and those with normal endosperm were studied without treatment and after treatment by the Bocchi technology (Bocchi Partnership, 1983). The composition and nutrient content are presented in Table 2.

In experiment III, maize + extracted soybean and maize + extracted sunflower mixtures were studied without treatment and after extrusion. The composition and nutrient content of concentrate mixtures are shown in Table 3. The basic materials were ground separately, but the maize + soybean and maize + sunflower mixtures were extruded together.

Schedule of the experiment

Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14

Weighing — Monday — Wednesday — Friday

Prefeeding period

Sample collection phase
chymus and faeces collection

Cr-NDF feeding

Schedule of chymus collection

Monday	7:00	11:00	15:00	19:00	
Tuesday					
Wednesday	8:20	12:20	16:20	20:20	
Thursday					
Friday	9:40	13:40	17:40		21:40

Fig. 1. Feeding of the experimental animals and the rotation of chymus and faecal sample collection

Table 1

Composition and nutrient content of concentrate mixtures containing different cereals (Experiment I)

Feed	1	2	3
	Treatments		
Components, %			
Triticale (Tewo)	96.0	48.0	48.0
Wheat (Mv 15)	–	48.0	–
Maize	–	–	48.0
Swine premix*	4.0	4.0	4.0
Total:	100.0	100.0	100.0
Nutrient content, g/kg			
Organic matter	854	855	863
Crude protein	169	160	138
Crude fat	19	26	33
Crude fibre	31	28	30
Crude ash	47	42	42
N-free extract	615	640	661
Starch	490	493	522
DE, MJ/kg	15.05	15.29	15.38
Amino acid composition (in % of dry matter)			
ALA	0.61	0.57	0.62
ARG	0.70	0.72	0.43
ASP	1.04	0.95	0.76
CYS	0.29	0.28	0.17
GLU	6.23	6.05	4.28
GLY	0.74	0.75	0.44
HIS	0.41	0.40	0.30
ILE	0.47	0.50	0.34
LEU	1.08	1.01	1.15
LYS	0.61	0.48	0.35
MET	0.26	0.25	0.19
PHE	0.71	0.68	0.48
PRO	1.23	1.31	1.05
SER	0.69	0.67	0.49
THR	0.50	0.49	0.39
TYR	0.37	0.38	0.35
VAL	0.67	0.65	0.48

* One kg of premix contains: 16.17% Ca, 4.35% P, 9.70% NaCl, 3.80% Na, 131,250 IU vitamin A, 30,625 IU vitamin D_3, 350 mg vitamin E, 21.87 mg vitamin K_3, 8.2 mg vitamin B_1, 28.87 mg vitamin B_2, 20.78 mg vitamin B_6, 0.23 mg vitamin B_{12}, 65.62 mg nicotinic acid, 1032.5 mg BHT antioxidant, 1381.25 mg Zn, 1381.25 mg Fe, 1062.50 mg Cu, 690.62 mg Mn, 20.72 mg I, 2.12 mg Se

Table 2

Composition and nutrient content of concentrate mixtures containing maize treated by the Bocchi technology (Experiment II)

Feed	Hybrid maize			
	Normal		Waxy	
	(1) Untreated	(2) Treated by the Bocchi procedure	(3) Untreated	(4) Treated by the Bocchi procedure
Components, %				
Maize meal	96.0	96.0	96.0	96.0
Swine premix*	4.0	4.0	4.0	4.0
Total:	100.0	100.0	100.0	100.0
Nutrient content, g/kg				
Organic matter	954	960	959	955
Crude protein	112	106	136	136
Crude fat	43	45	40	48
Crude fibre	28	23	27	24
Crude ash	46	40	40	45
N-free extract	771	785	756	769
Starch	700	649	608	611
DE, MJ/kg	15.90	16.01	16.04	16.42
Amino acid composition (in % of dry matter)				
ALA	0.70	0.67	0.71	0.73
ARG	0.43	0.41	0.44	0.45
ASP	0.60	0.55	0.61	0.63
CYS	0.10	0.09	0.12	0.11
GLU	1.90	1.81	1.93	1.98
GLY	0.29	0.33	0.30	0.35
HIS	0.21	0.23	0.25	0.26
ILE	0.24	0.25	0.32	0.37
LEU	1.07	1.01	1.22	1.34
LYS	0.26	0.29	0.27	0.31
MET	0.18	0.17	0.17	0.17
PHE	0.42	0.41	0.42	0.44
PRO	0.80	0.77	0.91	1.04
SER	0.44	0.40	0.46	0.47
THR	0.30	0.28	0.33	0.35
TYR	0.31	0.27	0.33	0.34
VAL	0.39	0.36	0.45	0.49

*For the composition of premix, see footnote to Table 1.

Table 3

Composition and nutrient content of extruded and untreated concentrate mixtures (Experiment III)

Feed	Experimental groups			
	(1) untreated	(2) extruded	(4) untreated	(5) extruded
	maize + soybean		maize + sunflower	
Components, %				
Maize meal	76.8	76.8	76.8	76.8
Extracted soybean meal	19.2	19.2	–	–
Extracted sunflower meal	–	–	19.2	19.2
Swine premix*	4.0	4.0	4.0	4.0
Total:	100.0	100.0	100.0	100.0
Nutrient content, g/kg				
Organic matter	950	951	929	948
Crude protein	185	184	198	185
Crude fat	40	23	32	27
Crude fibre	45	29	71	59
Crude ash	49	50	71	51
N-free extract	681	715	627	676
Starch	488	546	463	502
DE, MJ/kg	15.59	15.71	15.12	15.22
Amino acid composition (in % of dry matter)				
ALA	0.67	0.58	0.83	0.81
ASP	1.13	0.85	1.17	1.29
CYS	0.13	0.12	0.12	0.13
GLU	2.64	2.28	3.37	3.42
GLY	0.40	0.38	0.75	0.74
HIS	0.31	0.31	0.39	0.36
ILE	0.47	0.41	0.44	0.47
LEU	1.12	1.03	1.16	1.23
LYS	0.76	0.70	0.45	0.46
MET	0.15	0.14	0.28	0.24
PHE	0.50	0.46	0.58	0.73
PRO	0.69	0.89	1.00	1.04
SER	0.61	0.51	0.72	0.75
THR	0.42	0.37	0.52	0.53
TYR	0.25	0.19	0.37	0.29
VAL	0.51	0.44	0.67	0.60

*For the composition of premix, see footnote to Table 1.

Laboratory analyses

The chymus and faecal samples collected during the experiments were frozen at –18 °C, then lyophilised.

The dry matter, crude protein, crude fibre, crude fat and crude ash content of the feeds, and the dry matter and crude protein content of the chymus and faecal samples were determined according to Hungarian Standard MSz 6830. For determination of the amino acid content the feed, chymus and faecal samples were hydrolysed with 6N hydrochloric acid at 110 °C for 24 h. Amino acids were separated by ion-exchange column chromatography, using an Aminochrom II type instrument. To enable a reliable determination of methionine and cystine, oxidation with performic acid was applied, and the oxidised products Cys (O_3H) and Met (O) were measured (Hungarian Feed Codex, 1990).

The concentration of chromic oxide used as particle marker was determined by the method of Fenton and Fenton (1979). The starch content of feeds as well as chymus and faecal samples was determined with the help of a Boehringer "UV test" after the enzymatic digestion described by Seidler et al. (1988).

Statistical analysis

Mathematical evaluation of the data was done by one-way analysis of variance using a randomised block design (Sváb, 1973). The significance of differences between pairs of the test parameters was evaluated by Student's *t*-test.

Results and discussion

Experiment I

In this experiment the digestibility of nutrients of the "Tewo" triticale as a newly improved variety was studied and compared to values obtained when this triticale variety was fed in combination with other cereals.

The ileal and faecal digestibility values of the nutrients are presented in Table 4. The ileal and faecal digestibility of dry matter was much higher in groups 2 and 3 than in group 1 fed triticale alone. The ileal digestibility of crude protein was 71.9% in the group fed triticale alone, and was significantly improved by maize (77.5%, group 3) and wheat (79.1%, group 2) supplementation.

Similar changes were observed in the ileal digestibility values of amino acids. As compared to group 1, the feeding of cereal combinations (groups 2 and 3) resulted in a significant improvement in the ileal digestibility of cystine, threonine, methionine, isoleucine, leucine, tyrosine, and valine, while the ileal digestibility of lysine increased significantly in group 2 only.

Table 4

Ileal and faecal digestibility (%) of nutrients of concentrate mixtures containing triticale (Experiment I)

Nutrient	Experimental groups					
	(1) Triticale		(2) Triticale + wheat		(3) Triticale + maize	
	ID	FD	ID	FD	ID	FD
Dry matter	70.4 ± 2.6[2,3]	82.7 ± 0.6[2,3]	78.7 ± 1.6[1]	87.0 ± 0.5[1]	79.9 ± 1.0[1]	86.6 ± 1.0[1]
Crude protein	71.9 ± 2.2[2,3]	81.7 ± 1.9[2]	79.1 ± 2.1[1]	85.4 ± 1.4[1]	77.5 ± 3.0[1]	82.8 ± 2.4
Starch	94.9 ± 1.5	98.7 ± 0.1	95.6 ± 1.2	98.8 ± 0.0	96.5 ± 0.5	99.1 ± 0.1
Amino acids						
CYS	80.6 ± 1.4[2,3]	91.5 ± 1.1[2]	89.8 ± 1.6[1,3]	96.6 ± 0.6[1]	85.6 ± 1.3[1,2]	92.9 ± 3.7
ILE	69.6 ± 2.3[2,3]	80.3 ± 2.6[2,3]	85.5 ± 2.8[1,3]	91.8 ± 1.3[1,3]	78.3 ± 3.1[1,2]	85.9 ± 2.0[1,2]
LEU	70.1 ± 2.6[2,3]	83.3 ± 1.4[2,3]	83.4 ± 1.8[1]	93.1 ± 0.8[1]	84.4 ± 4.7[1,2]	91.8 ± 1.4[1]
LYS	75.0 ± 1.6[2]	80.7 ± 1.5	79.2 ± 2.3[1]	85.5 ± 1.6[1,3]	78.6 ± 3.4	79.6 ± 1.0[2]
MET	74.0 ± 0.6[2,3]	84.0 ± 2.6[2]	86.2 ± 2.7[1,3]	90.3 ± 1.8[1,3]	78.6 ± 2.4[1,2]	85.2 ± 0.9[2]
THR	62.9 ± 2.8[2,3]	77.4 ± 1.3[2,3]	78.5 ± 3.0[1]	90.6 ± 1.5[1,3]	75.6 ± 3.7[1]	87.1 ± 1.8[1,2]
TYR	73.3 ± 3.3[2,3]	84.1 ± 2.1[2,3]	83.6 ± 0.5[1,3]	91.1 ± 1.6[1,3]	81.9 ± 1.0[1,2]	87.6 ± 1.9[1,2]
VAL	57.6 ± 2.6[2,3]	81.4 ± 2.4[2,3]	81.7 ± 2.0[1,3]	91.0 ± 1.5[1,3]	78.0 ± 2.4[1,2]	85.4 ± 1.6[1,2]

The group numbers shown in superscript indicate significant differences between treatments ($P < 0.05$)
ID = ileal digestibility; FD = faecal digestibility

The differences found in the ileal digestibility values of crude protein and amino acids can be explained by the findings of Buraczewska et al. (1987) who compared several varieties of barley. They arrived at the general conclusion that an increase in the N content of cereals may result in improved ileal digestibility of amino acids, and that the digestibility of protein and amino acids may be influenced by the carbohydrate content of the cell wall.

Sauer et al. (1977), Taverner et al. (1981) and Adeola et al. (1986) found that triticale contains more albumin and globulin but less gluten and insoluble protein than wheat. This is why triticale protein has higher lysine and lower glutamic acid content that wheat protein. Differences in the ileal digestibility of the N content of cereals are not completely consistent with those in the digestibility of most amino acids. In the case of wheat and triticale, the digestibility of N is usually higher than that of amino acids. This may be related to low protein solubility. At the same time, the digestibility of maize N was found to be lower than that of amino acids. According to findings of the above-mentioned authors, in cereal mixtures the digestibility of amino acids increases more than that of N.

Taverner et al. (1981) compared wheat, maize and triticale in terms of nutrient digestibility. The apparent ileal digestibility of lysine in several wheat varieties, maize varieties and triticale was found to be 62–81%, 71–82% and 81.0%, respectively. In the present study, the ileal digestibility of lysine was 75.0% in triticale, and it increased to 79.2% and 78.6% in 1:1 mixtures of triticale with wheat and maize, respectively. It can be established that the feeding of triticale in combination with either wheat or maize causes a very favourable change in lysine digestibility. In their experiments using pigs provided with a small intestinal cannula, Rakowska et al. (1990) compared the digestibility of triticale with that of barley and rye. For the essential amino acids lysine and threonine and for starch they obtained ileal digestibility values consistent with those found in this study.

The feeding of cereal combinations containing wheat or maize improved also the faecal digestibility data of amino acids; however, the improvement was not as large as that found for the ileal digestibility values.

The differences found between the ileal and faecal digestibility values of nutrients of the diets fed to the three groups are summarised in Table 5. These differences were smaller in groups 2 and 3 than in group 1. In the case of protein and amino acids, the difference between the two digestibility values is due to the fact that the absorption of these nutrients is completed by the time that the digesta reach the end of the ileum, and in the postileal segment of the gut microbial protein synthesis takes place simultaneously with proteolysis. That protein has an amino acid composition typical of microbial protein and different from that of dietary protein. Microbes degrade amino acids into ammonia and amines which become absorbed and then excreted in the urine (Zebrowska et al., 1977; Just et

al., 1981). Hermann (1988) and Schröder et al. (1988) accept measurements extending down to the end of the small intestine because that is the most distal intestinal segment where amino acids of dietary origin, available for protein synthesis during intermediary metabolism, still become absorbed.

Table 5

Differences between the ileal and faecal digestibility percentages of nutrients of concentrate mixtures containing triticale (Experiment I)

Nutrient	Experimental groups		
	1	2	3
Dry matter	12.3	8.3	6.7
Crude protein	9.8	6.3	5.3
Starch	3.8	3.2	2.6
Amino acids			
CYS	10.9	6.8	7.3
ILE	10.7	6.3	7.6
LEU	13.2	9.7	7.4
LYS	5.7	6.3	1.0
MET	10.0	4.1	6.6
THR	14.5	12.1	11.5
TYR	10.8	7.5	5.7
VAL	23.8	9.3	7.4

The present experiments conducted with cereals have shown that following digestion of the protein of "Tewo" triticale fed alone some of the amino acids become absorbed, which is expressed by the values of ileal digestibility. When feeding the combinations containing wheat or maize the digestibility values of amino acids change: the absorption of some amino acids will increase, that of others will become less efficient or possibly remain the same. During digestion, the protein contained in each feed combination will yield amino acid quantities corresponding to its composition, and the availability of individual amino acids for meeting the animal's requirements will be determined by their digestibility. The amino acid compositions indicated in practical feeding tables differ from the ileal digestibility values; therefore, in the light of the present studies the values shown in such tables should be regarded as a guideline only.

Ileal digestibility data apply to the digestion of nutrients taken up by the animal and to the absorption of its components. Under practical conditions, such data enable a more accurate assessment of how and to what extent the requirements are met; thus, they can be a valuable aid in the formulation of feed compositions.

Experiment II

As a result of treatment by the Bocchi technology, the ileal digestibility values of the two maize hybrids changed in a dissimilar manner (Table 6).

After Bocchi treatment, the ileal digestibility of crude protein increased in the case of both the normal and the waxy hybrid, while that of starch did not show appreciable changes.

In the case of the normal hybrid, the ileal digestibility of lysine increased from 58.5% to 72.7% as a result of treatment. The digestibility of cystine, valine and isoleucine also increased, even if to a lesser extent. Of the amino acids of the waxy hybrid, the digestibility of isoleucine, leucine, methionine, tyrosine and valine significantly increased as a result of heat treatment, while that of threonine, cystine and lysine changed only slightly. Treatment of the normal hybrid substantially improved the faecal digestibility values of all amino acids but methionine. At the same time, treatment of the waxy hybrid reduced the faecal digestibility values of methionine, tyrosine, threonine, cystine and lysine.

The differences found between the two maize hybrids studied in this experiment were as follow. While the starch of the maize hybrid of normal endosperm consists of 25% amylose and 75% amylopectin, that of the waxy hybrid is built up exclusively from amylopectin (Rosa et al., 1977). The crude protein content and amino acid composition of the normal hybrid were typical of a medium-quality hybrid, but its starch content was lower than the values reported by Sauer et al. (1977) and Perez and Aumaitre (1979), and came closer to the starch content given by Seidler et al. (1988). It should be mentioned that in the present study the starch determination method described by Seidler et al. (1988) was used. The crude protein content of the waxy hybrid was higher than the values reported in the above-cited papers; however, there was hardly any difference in the amino acid composition. The above-mentioned authors also found a lower starch content in the waxy maize than in the normal hybrid. Besides protein, the energy level of the diet, which is determined decisively by the carbohydrate content, is one of the most important factors in the fattening of animals. Carbohydrates constitute a heterogeneous group of compounds, the intestinal transformation of which differs both in the type of process involved and the site of degradation. Highly soluble carbohydrates (starch and sugar) are degraded mainly in the small intestine by the enzymes produced by the organism, whereas degradation of the cell wall material is done by an energetically less efficient microbial fermentation essentially in the postileal segment of the intestine (Schulz, 1990).

In similar trials conducted on pigs provided with a cannula, Sauer et al. (1977) and Freire et al. (1988) found that in the case of an average maize hybrid there was hardly any difference between the ileal and faecal digestibility of the essential amino acids lysine and methionine; at the same time, marked differences

Table 6

Ileal and faecal digestibility (%) of nutrients of normal and waxy maize hybrids (Experiment II)

Nutrient	Experimental groups							
	Normal hybrid maize				Waxy hybrid maize			
	(1) Untreated		(2) Treated by the Bocchi procedure		(3) Untreated		(4) Treated by the Bocchi procedure	
	ID	FD	ID	FD	ID	FD	ID	FD
Dry matter	79.0 ± 1.6[2]	85.7 ± 1.0[2]	84.7 ± 0.7[1]	87.7 ± 1.1[1]	78.7 ± 0.7	85.4 ± 1.0[4]	78.7 ± 1.5	87.4 ± 1.3[3]
Crude protein	69.2 ± 3.0	75.9 ± 2.6[2,3]	73.5 ± 2.7	82.4 ± 1.8[1]	65.8 ± 3.8	80.3 ± 0.9[1,4]	69.5 ± 7.0	83.2 ± 1.3[3]
Starch	97.5 ± 0.7	99.3 ± 0.0	98.6 ± 0.2	99.5 ± 0.0	97.8 ± 0.1	99.2 ± 0.1	97.7 ± 0.2	99.4 ± 0.1
Amino acids								
CYS	72.9 ± 4.2[2,3]	68.1 ± 1.0[3,2]	84.2 ± 2.6[1]	81.8 ± 3.9[1]	80.8 ± 3.9[1]	82.0 ± 1.6[1,4]	80.7 ± 1.4	77.5 ± 1.1[3]
ILE	71.9 ± 3.5[2]	78.9 ± 1.6[3,2]	78.0 ± 4.3[1]	87.3 ± 2.1[1]	76.2 ± 2.7[4]	86.3 ± 1.2[1]	81.2 ± 4.1[3]	86.1 ± 1.6
LEU	84.5 ± 4.8	88.5 ± 1.2[3,2]	89.7 ± 1.9	93.7 ± 1.1[1]	88.3 ± 1.1[4]	93.4 ± 0.6[1]	92.4 ± 1.6[3]	93.3 ± 1.4
LYS	58.5 ± 3.2[2]	73.2 ± 3.4[2]	72.7 ± 3.3[1]	81.1 ± 2.3[1]	57.0 ± 4.2	75.4 ± 3.9	60.5 ± 4.0	73.4 ± 3.5
MET	75.9 ± 3.8[3]	81.3 ± 1.5[3]	76.0 ± 5.7	83.3 ± 1.3	67.7 ± 3.4[1,4]	76.0 ± 2.6[1]	74.1 ± 4.4[3]	73.9 ± 6.8
THR	59.1 ± 2.3	75.4 ± 3.0[2,3]	62.6 ± 3.2	85.5 ± 2.0[1]	62.8 ± 2.8	81.7 ± 2.2[1]	64.5 ± 7.0	80.7 ± 2.6
TYR	57.2 ± 4.1	86.8 ± 1.2[2]	59.5 ± 4.0	89.0 ± 1.2[1]	52.8 ± 2.4[4]	88.0 ± 1.7	72.0 ± 5.3[3]	86.0 ± 1.3
VAL	67.8 ± 3.4[2]	78.7 ± 1.6[3,2]	74.4 ± 3.1[1]	86.8 ± 1.5[1]	70.8 ± 3.6[4]	85.5 ± 1.4[1]	77.0 ± 4.0[3]	84.2 ± 2.3

The group numbers shown in superscript indicate significant differences ($P < 0.05$) between the waxy and the treated waxy, between the normal and the treated normal, as well as between the untreated normal and the waxy maize hybrids; ID = ileal digestibility; FD = faecal digestibility

were found for threonine and cystine as well as for dry matter and crude protein, and about 98% of the starch became digested by the end of the ileum. They also stated that the type of starch does not affect the fattening performance of pigs. Citing several other authors, Rosa et al. (1977) confirm that the starch of waxy maize is more sensitive to enzymatic hydrolysis than that of normal maize; however, this is not reflected in the fattening performance of animals.

Leeuwen et al. (1987) studied the digestibility of an average maize sample in cannulated pigs, and found the ileal digestibility of crude protein, lysine, methionine and threonine 70%, 57%, 82% and 61%, respectively. The results obtained in this study for both maize hybrids show good agreement with these values. The digestibility coefficients determined by faecal analysis by Leeuwen et al. (1987) tended to increase in a similar manner as in this study.

The differences obtained in the digestibility coefficients by ileal and faecal analysis are summarised in Table 7. In general, it can be stated that the differences found between the untreated normal and waxy hybrids in praecaecal and postileal digestibility values are bigger than after treatment by the Bocchi procedure. Of the amino acids, the biggest difference was found for tyrosine, threonine, lysine and valine, in contrast to the results of Sauer et al. (1977), who found a substantial difference for threonine only, although they stated that the ileal amino acid digestibility coefficients of cereals are lower than those obtained by faecal analysis. Hartog et al. (1988) compared extruded and untreated maize, constituting 60% of a concentrate mix, in cannulated weaned piglets. Extrusion was found to cause a slight improvement in the ileal and faecal digestibility coefficients of nutrients, including amino acids. At the same time, the differences between the ileal and the faecal digestibility coefficients usually exceeded 10%. In the experiment conducted by Poel et al. (1990), the treatment of maize markedly improved the *in vitro* digestibility; however, in the experiment involving weaned piglets no change was found in the ileal digestibility of any nutrients except organic matter, N-free extract and, of the amino acids, cystine and proline.

Cystine was the only amino acid for which the difference between ileal and faecal digestibility (Table 7) based upon chymus analysis was higher than that determined by faecal analysis. In the case of the waxy hybrid, the difference between ileal and faecal digestibility of crude protein was 14.5% and 13.7% for the untreated and the treated samples, while for the normal hybrid the corresponding values were 6.7% and 8.9%. Of the amino acids, tyrosine, threonine, lysine, and valine showed the biggest difference in faecal digestibility as compared to the results obtained by ileal analysis.

Table 7

Differences between the ileal and faecal digestibility percentages of nutrients of maize hybrids (Experiment II)

Nutrient	Normal maize hybrid		Waxy maize hybrid	
	(1) Untreated	(2) Treated by the Bocchi procedure	(3) Untreated	(4) Treated by the Bocchi procedure
Dry matter	6.7	3.0	6.7	8.7
Crude protein	6.7	8.9	14.5	13.7
Starch	1.8	0.9	1.4	1.7
Amino acids				
CYS	–4.8	–2.4	–1.2	–3.2
ILE	7.0	9.3	10.1	4.9
LEU	4.0	4.0	5.1	0.9
LYS	14.7	8.4	18.4	12.9
MET	5.4	7.3	8.3	–0.2
THR	16.3	22.9	18.9	16.2
TYR	29.6	29.5	35.2	14.0
VAL	10.9	12.4	14.7	7.2

Experiment III

The changes that occurred in the ileal and faecal digestibility of starch, crude protein and amino acids when feeding extruded mixtures of maize + soybean and maize + sunflower are presented in Table 8.

Between the extruded and untreated varieties of the maize + extracted soybean mixture there was a significant ($P < 0.001$) difference in the ileal digestibility of dry matter and crude protein; the latter increased from 61.6% to 70.3%. Of the essential amino acids, the digestibility of lysine increased from 70.7% to 82.7%, that of methionine from 60.1% to 72.0%, and that of threonine from 41.1% to 59.4%, but the ileal digestibility of the other amino acids also underwent a significant ($P < 0.001$) increase as a result of extrusion.

When the maize + extracted sunflower mixture was fed, the digestibility of not all nutrients changed as a result of extrusion. The ileal digestibility of crude protein significantly increased (from 80.6% to 84.5%); as regards the amino acids, significant improvement occurred in the ileal digestibility of lysine (from 61.4% to 72.3%), cystine (67.7% to 74.1%), and leucine (84.4% to 91.1%), while the other amino acids showed only slight changes in ileal digestibility.

Extrusion caused a significant improvement also in the faecal digestibility of nutrients of the soybean-containing diet, with the exception of cystine and starch. In the case of the maize + sunflower mixture, significant improvement oc-

Table 8

Ileal and faecal digestibility (%) of nutrients of extruded and untreated concentrate mixtures (Experiment III)

Nutrient	Experimental groups							
	Maize-extracted soybean				Maize-extracted sunflower			
	(1) Untreated		(2) Treated by the Bocchi procedure		(3) Untreated		(4) Treated by the Bocchi procedure	
	ID	FD	ID	FD	ID	FD	ID	FD
Dry matter	72.3 ± 1.3[2]	83.6 ± 0.2[2]	81.1 ± 1.1[1]	88.1 ± 0.6[1]	77.3 ± 0.7[4]	82.8 ± 0.6[4]	80.2 ± 1.0[3]	86.1 ± 0.3[3]
Crude protein	61.6 ± 2.2[2]	81.8 ± 1.7[2]	70.3 ± 4.7[1]	88.2 ± 0.9[1]	80.6 ± 1.8[4]	84.0 ± 0.9	84.5 ± 1.1[3]	85.2 ± 2.9
Starch	97.0 ± 0.5[2]	98.8 ± 0.1[2]	98.3 ± 0.1[1]	99.4 ± 0.0[1]	97.7 ± 0.4	98.9 ± 0.1[4]	97.9 ± 0.1	99.3 ± 0.1[3]
Amino acids								
CYS	66.1 ± 6.2	66.8 ± 4.5	70.6 ± 4.0	71.3 ± 3.0	67.7 ± 2.1[4]	80.1 ± 2.7[4]	74.1 ± 4.1[3]	88.6 ± 1.1[3]
ILE	62.9 ± 3.7[2]	73.0 ± 3.1[2]	80.5 ± 1.9[1]	83.0 ± 0.9[1]	78.3 ± 4.5	80.5 ± 2.9	85.2 ± 2.9	88.4 ± 1.2
LEU	70.3 ± 1.9[2]	76.6 ± 2.3[2]	84.8 ± 1.4[1]	86.9 ± 0.7[1]	84.4 ± 2.3[4]	92.8 ± 1.2	91.1 ± 1.7[3]	93.8 ± 0.8
LYS	70.7 ± 2.0[2]	77.4 ± 3.1[2]	82.7 ± 1.8[1]	84.7 ± 2.0[1]	61.4 ± 4.8[4]	76.0 ± 2.4[4]	72.3 ± 3.0[3]	84.5 ± 2.3[3]
MET	60.1 ± 4.5[2]	55.7 ± 1.7[2]	72.0 ± 5.8[1]	74.6 ± 3.8[1]	79.7 ± 2.8	92.9 ± 0.2[4]	84.3 ± 3.3	95.2 ± 0.7[3]
THR	41.1 ± 4.1[2]	66.8 ± 2.7[2]	59.4 ± 4.0[1]	78.1 ± 2.6[1]	78.1 ± 2.4	83.3 ± 1.7[4]	80.6 ± 2.3	88.6 ± 1.2[3]
TYR	27.9 ± 5.0[2]	69.7 ± 2.3[2]	43.0 ± 2.4[1]	76.3 ± 2.1[1]	79.4 ± 2.3	84.0 ± 0.1[4]	80.2 ± 5.2	88.7 ± 0.7[3]
VAL	49.7 ± 3.0[2]	69.4 ± 2.9[2]	70.2 ± 3.4[1]	77.5 ± 1.8[1]	80.1 ± 2.5	84.9 ± 2.3[4]	81.9 ± 2.9	88.4 ± 2.1[3]

The group numbers shown in superscript indicate significant differences ($P < 0.05$) between the untreated groups and the groups fed extruded concentrate mixtures; ID = ileal digestibility; FD = faecal digestibility

curred in the faecal digestibility coefficients of all nutrients except dry matter, crude protein, and the amino acids isoleucine and leucine.

The differences between the ileal and faecal digestibility values of the untreated and extruded varieties of the maize + soybean and maize + sunflower mixtures are shown in Table 9. With the only exception of cystine, the difference between the ileal and faecal digestibility of all nutrients of the untreated soybean-containing concentrate mix was bigger than that found for the extruded diet. It should be noted, however, that the ileal digestibility of methionine was higher than its faecal digestibility. The tendency was less clear in the sunflower experiment. The difference between the ileal and faecal digestibility of dry matter was the same for the untreated and the extruded diet, while for protein, sulphur-containing amino acids, leucine and lysine still the untreated diet gave a bigger difference.

Table 9

Differences between the ileal and faecal digestibility percentages of nutrients of extruded and untreated concentrate mixtures (Experiment III)

Nutrient	I (maize + soybean)		II (maize + sunflower)	
	(1) untreated	(2) extruded	(3) untreated	(4) extruded
Dry matter	11.3	7.0	5.5	5.9
Crude protein	20.2	17.9	3.4	0.7
Starch	1.8	1.1	1.2	1.4
Amino acids				
CYS	0.7	0.7	20.4	14.5
ILE	10.1	2.5	2.2	3.2
LEU	6.3	2.1	8.4	2.7
LYS	6.7	2.0	14.6	12.2
MET	–4.4	2.6	13.2	10.9
THR	25.7	18.7	5.2	8.0
TYR	–	–	4.6	8.5
VAL	19.7	7.3	4.8	6.5

The results presented above seem to support the statement that extrusion of the soybean-containing diet markedly improved the digestibility of protein and amino acids. However, the effect of extrusion also depends on the starting material. Thus, e.g. the ileal digestibility of nutrients of the soybean mix was much lower than that of the sunflower mix, irrespective of whether the untreated or the extruded diet was studied. At the same time, extrusion caused a bigger improvement in the digestibility of nutrients of the soybean mix than in that of the sunflower mix. Further analysis of results of the soybean experiment showed that the

difference between the ileal and faecal digestibility of cystine was the same for the untreated and the extruded diet. The faecal digestibility of methionine, unlike that of the other amino acids, was lower, and this resulted in the negative value for the untreated diet. Rudolph et al. (1983) made a similar statement after comparative studies on the extrusion of different soybean products. Methionine is utilised by the large intestinal microflora, and this causes the bigger decrease in faecal concentration. This may account for the difference obtained in our soybean experiment between the ileal and faecal digestibility of methionine. To confirm the hypothesis that amino acids having passed the terminal ileum will not be utilised, Rudolph et al. (1983) infused protein and amino acids into the small intestine of pigs. They found that the N absorbed from these compounds was always excreted in the urine. From this finding they concluded that amino acids present in the large intestine have negligible nutritive value for the pig.

Vandergrift et al. (1983) and Jørgensen et al. (1984) subjected raw and defatted soybean to hydrothermic treatment of different duration, and subsequently studied the changes occurring in the ileal and faecal digestibility of its protein and amino acids in pigs fitted up with a small intestinal T-cannula. Hydrothermic treatment of increasing duration doubled the digestibility of proteins and amino acids as determined by chymus analysis, while the differences found by faecal examination, though existent, were far smaller than those obtained by ileal analysis. The ileal and faecal digestibility values obtained in the present study were lower than those reported by the above-cited authors. This may be due to the fact that the above-cited authors used soybean as exclusive protein source in addition to N-free maize starch, while in the present experiments the digestibility of the maize + soybean combination was measured. Thacker et al. (1984) fed barley and soybean meal, and obtained similar ileal digestibility values for amino acids as those found in the present study.

The effectiveness of extrusion is supported by the results obtained by Hartog et al. (1988) in an experiment conducted with piglets weighing 20 kg. The piglets, provided with a re-entrant (ileal-caecal) cannula were fed a mixture consisting of components of which only maize had been extruded. The ileal digestibility of nutrients increased; however, the results of faecal analysis showed no effect whatever attributable to the treatment.

The ileal and faecal digestibility values obtained in the sunflower experiment were higher than those found in the soybean trial; however, the difference between the digestibility values determined by chymus and faecal analysis decreased. In a similar experiment, Thacker et al. (1984) used sunflower combined with barley, and they also obtained higher digestibility values. Both in their and in our experiment, the lysine content was lower in the sunflower mixture than in the soybean diet. Of the amino acids, lysine had the lowest digestibility by both ileal

and faecal analysis. In our experiment, however, extrusion significantly ($P < 0.01$) improved the ileal digestibility of lysine. In addition, methionine was absorbed much more efficiently in this experiment than when the soybean-containing mixture was fed. This may be explained by the fact that the sunflower mixture contained almost twice as much methionine as the one containing soybean. In the opinion of Thacker et al. (1984), soybean can be replaced by sunflower if the latter is supplemented with lysine, and if the difference in available amino acid content is compensated for by the addition of cereals.

According to Sauer and Ozimek (1986), of the amino acids of sunflower meal lysine has the lowest digestibility. This is in agreement with our findings. The difference between ileal and faecal digestibility may depend on the quantity of carbohydrates fermented in the large intestine. Therefore, it is important whether the protein source to be studied is fed in combination with a cereal or with e.g. maize starch. Namely, the aleurone cells forming the outer layer of the endosperm in cereal grains and the thick cellulose cell wall hinder digestion.

Misir and Sauer (1982) suggested that microbial activity in the large intestine is determined by the availability of energy substrate. In their trial conducted on pigs, they compared potato starch with an easily digestible maize starch, and found that the level of microbial activity increased as a result of the higher amount of undigested starch reaching the large intestine. In their opinion, the net synthesis or absorption of amino acids in the large intestine of pigs is influenced by the composition of protein source and the amount of energy substrate available there. Heavier pigs can digest fibre more efficiently than lighter ones (Just et al., 1979); thus, the former can use more energy for microbial protein synthesis in the large intestine. Similar results were obtained by Jørgensen et al. (1984), who studied sunflower as exclusive protein source, fed together with maize starch. The digestibility coefficients found by them are the same as those obtained in the present study. In their opinion, substantial differences existed among the protein sources, resulting in differences in amino acid availability which, in turn, influences the amino acid supply of pigs. They also suggested that faecal analysis usually overestimates the availability of essential amino acids, including lysine and threonine which are limiting amino acids in pig diets.

In the present trial, 97–98% of the starch became digested by the time it reached the terminal ileum, irrespective of whether or not extrusion was applied. These values are fully consistent with those obtained by Schulz et al. (1988) who, studying different cereals and their combinations, found the digestibility of starch to be 94–98% in the intestinal segments proximal to the caecum. Fadel et al. (1989) extruded concentrate mixtures of extracted soybean and barley under different conditions of temperature and moisture. They obtained 94.8% ileal starch digestibility for the untreated mixture and 96.9% and 98.6%, respectively, for the

treated mixtures. In their opinion, as cereals are a major component of concentrate mixtures and since most of their carbohydrates is constituted by starch, a 2–4% improvement of digestibility is not negligible at all.

Conclusions

Based upon its established crude protein content and amino acid composition, Tewo triticale can be a useful component of pig diets. This is supported by the results of trials involving pigs provided with an ileal cannula. When feeding Tewo triticale in 1:1 mixtures with either wheat or maize, the ileal digestibility of protein, amino acids and starch is so favourable that Tewo triticale can by all means be recommended for use as an alternative cereal. The results of previous N balance studies also indicate that the Tewo variety exerts a favourable influence on N retention in growing pigs (Szelényi-Galántai et al., 1994).

In the present experiments, treatment of normal hybrid maize by the Bocchi procedure caused a significant improvement in the ileal digestibility of lysine, cystine, isoleucine, valine, and dry matter. Bocchi treatment also markedly improved the faecal digestibility of all nutrients except methionine.

No marked variety- or treatment-related differences were found in the digestibility of starch. This indicates that starch is utilised in the digestive system of pigs even without treatment.

Extrusion as a feed processing technology improved the crude protein digestibility of mixed feeds and the absorption of amino acids from intestinal segments proximal to the caecum in the case of both the soybean- and the sunflower-containing combinations. Feed composition is very important for the improving effect exerted by extrusion on digestibility. If, depending on the protein source, supplementation with synthetic amino acids is necessary, this fact should be taken into consideration.

Extrusion increased the ileal digestibility of crude protein of the soybean mix by 14% while that of the sunflower mix by only 5%. This should be kept in mind when formulating rations. The extrusion process is indicated for use primarily where the value of feed thus saved is commensurate with the additional technological costs. In the practice, its use is recommended first of all in intensive livestock management or in the rearing of young animals.

A further benefit of extrusion is that the resulting higher ileal digestibility of amino acids allows us to diminish the amount of dietary protein fed, which, in turn, will reduce the animals' nitrogen excretion and, thus, environmental pollution in and around livestock holdings.

The results presented in this paper also indicate that the ileal digestibility coefficients of amino acids enable a more accurate assessment of the nutritive value of proteins and a more exact judgement of how closely the animals' requirements are met. This is supported by the results of Sauer (1993) who, when formulating concentrate mixes on the basis of the ileal digestibility of protein, found a correlation of $r = 0.76$ between the body weight gain and digestible protein intake of pigs, as well as a correlation of $r = -0.87$ between the body weight gain and the amount of feed used for 1 kg of gain.

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COMPARISON OF SOME BLOOD PARAMETERS OF CAPTURED AND FARMED RED DEER (*CERVUS ELAPHUS*) HINDS

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In studies on the adaptation of red deer (*Cervus elaphus*) to farm conditions, some blood parameters of 3- to 9-year-old captured and farmed hinds were compared. The test variables included blood plasma creatine kinase (CK), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) activities, the concentrations of glucose, urea, total protein (TP), serum cortisol, haemoglobin (Hb), and the blastogenic transformation of lymphocytes upon stimulation by the nonspecific mitogens *Phaseolus vulgaris* (PHA) and Concanavalin A (ConA) in the lymphocyte stimulation test (LST). The effects exerted by a probiotic preparation (Ascogen®) on the same parameters were studied in farmed deer. In hinds that had been kept under farm conditions for 10 months, CK activity was lower ($P < 0.05$) and blood urea concentration higher ($P < 0.001$) than at the time of capture, while their blood glucose concentration exceeded ($P < 0.01$) that of farmed hinds. Hinds treated with Ascogen® had lower ALT activity ($P < 0.001$) and showed a higher increase in PHA-induced blastogenic transformation of lymphocytes than the control hinds.

Key words: Alanine aminotransferase, blastogenic transformation of lymphocytes, cortisol, creatine kinase, glucose, haemoglobin, lactate dehydrogenase, total protein, urea, red deer

The keeping of red deer (*Cervus elaphus*) under farm-like conditions as a new management technology has gained ground rapidly all over the world in recent years. In Hungary, the intensive domestication of red deer was started a decade ago. The addition of captured feral deer has considerably enlarged farmed stocks of red deer.

Red deer kept in confinement in a closed space are exposed to permanent or temporary stress factors different from those occurring in the wild. Depending on

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the severity and duration of stress, higher living organisms try to maintain their internal balance by Cannon's alarm reaction or through the general adaptation syndrome (GAS) (Cannon, 1929; Selye, 1946).

During adaptation to a stressor, the organism responds with enhanced glucocorticoid production by the hypothalamus–pituitary–adrenal cortex system (Axelrod and Reisine, 1984), which diminishes the stressor-induced effects of numerous intracellular mediators and hormones (Munck et al., 1984). Through its immunosuppressive effect (Boumpas et al., 1991), cortisol increases the susceptibility of the organism to bacterial, viral, mycotic and parasitic diseases as well as to oncogenic factors (Laborit, 1991).

As stressors directly decrease the secretion of gonadotropin-releasing and gonadotropic hormones, they may give rise to reproductive disturbances (Chatterton, 1990).

Depending on individual stress susceptibility, red deer exposed to acute stress may develop haemorrhagic enteropathy and postcapture myopathy (McAllum, 1981, 1985). Muscle cell membrane damage caused by acute stress or trauma leads to a measurable increase in the activity of creatine kinase (CK) and lactate dehydrogenase (LDH) enzymes of skeletal muscle origin in the blood plasma (Rick, 1992).

Data on the normal biochemical and haematological values of farmed red deer have been reported by Wilson and Pauli (1982*a*, 1982*b*) and Reid and Towers (1985) from New Zealand. Presidente (1979) published a review on haematological parameters of Cervidae living in Australia. Kent et al. (1980) analysed blood serum constituents of killed red deer in England.

The biochemical and haematological effects of immobilisation with phenycyclidine and promazine (Seal et al., 1972) or with etorphine HCl and xylazine (Presidente et al., 1973) in white-tailed deer (*Odocoileus virginianus*), and those of immobilisation with xylazine in red deer (Cross et al., 1988) have been reported.

Franzmann et al. (1975) found significant differences between the degree of excitation and the serum 11-hydroxycorticosteroid levels of Alaskan moose (*Alces alces gigas*). Van Mourik et al. (1985) demonstrated considerable differences in the cortisol concentration of plasma samples collected from rusa deer (*Cervus rusa timoriensis*) after driving in and following xylazine and fentanyl azaperone immobilisation. In stress induction studies with adrenocorticotropic hormone (ACTH), Bubenik et al. (1990) measured the serum cortisol concentration of white-tailed deer. Fehér et al. (1994) compared the levels of stress hormones synthesized in the adrenal cortex of farmed and captured red deer.

The objective of this work was to collect data on physiological blood parameters related to the adaptation and stress susceptibility of red deer hinds. As the difference between feral conditions and farm management constitutes a perma-

nent stress factor for red deer, the stress-reducing effects of a probiotic preparation (Ascogen®, Chemoforma AG, Switzerland) were studied in farmed hinds.

Materials and methods

Three- to nine-year-old red deer hinds weighing 120–160 kg were used as follows.

Blood samples from captured hinds were taken at the time of capture with a net (on 25 January 1994), after immobilisation with a combination of xylazine and ketamine (Zomborszky and Sugár, 1990) (n = 10) and 10 months thereafter under farm conditions (n = 11), without the use of crush, using only gentle physical restraint.

Farmed hinds kept on the farm for 6 years and hinds that had been born on the farm were co-mingled and divided into two groups (treated group, n = 12; control group, n = 12). Blood samples from these hinds were taken in January 1995 and subsequently at a later stage of gestation, at the end of March, with the help of crush.

The hinds were kept on pasture, and an additional 1–2 kg of hay, 3–4 kg of silage and 1 kg of ground maize per animal were provided. Between the first and the second sampling (i.e. for a period of 75 days), the fodder of hinds of the treated group was supplemented with the probiotic preparation Ascogen® [Chemoforma AG, Switzerland; composition: thermolysed brewer's yeast (*Saccharomyces cerevisiae*, type B.F.T., E.10); yeast extract from *S. cerevisiae*: RNA fraction, specific nucleotides, organic acids; vitamins A, D_3, E, C, B complex, folic acid; trace elements; stabiliser], homogeneously mixed in the ground maize at a dose of 3 g/animal/day. This granulated formulation is a natural feed supplement containing yeasts, intermediary metabolites, nucleotides, vitamins, and minerals.

Blood samples were taken from the jugular vein within 1 h after the animals were driven in, using a disposable needle and syringe.

From samples treated with lithium-heparin as anticoagulant, the plasma was separated by centrifugation and assayed with a VetTest 8008 dry biochemical blood analyser (IDEXX Cergy Pantoise Cedex, Libourne, France) to determine the activities of creatine kinase (CK), alanine aminotransferase (ALT), lactate dehydrogenase (LDH) and the concentrations of blood glucose, urea and total protein (TP) using Eastman Kodak slides (Eastman Kodak Company for IDEXX Laboratories, Inc., One IDEXX Drive, Westbrook, Maine, U.S.A.).

Lymphocytes in blood samples treated with heparin and Hanks' solution containing 10% fetal serum were subjected to blastogenic transformation by the nonspecific mitogens *Phaseolus vulgaris* (PHA) and Concanavalin A (ConA) in the lymphocyte stimulation test (LST) (Outteridge et al., 1981).

Serum cortisol concentration of whole blood samples was determined by the radioimmunological method of Bodrogi and Fehér (1987). The intra- and interassay coefficients of variation were 8.2% and 13.3%, respectively.

Haemoglobin (Hb) concentration was measured in blood samples treated with dipotassium ethylenediamine tetraacetate (dipotassium EDTA), in a PS-5 type blood analyser (Medicor, Budapest, Hungary).

Analyses of variance and correlation were performed using a statistical programme package of SAS (1985).

Results

The biochemical, immunological and haematological values of captured and farmed red deer hinds are presented in Table 1.

Following capture with a net, a procedure known to be accompanied by acute stress, the investigation of parameters indicative of stress susceptibility revealed high positive correlations between the activities of CK and LDH (enzymes of mostly muscle origin), the activity of ALT (which is an enzyme of primarily hepatic origin), and the concentrations of serum cortisol ($r > 0.65$; $P < 0.05$). At the same time, the changes in blood glucose level were not consistent with the alterations measured in the parameters indicative of stress. In captured hinds CK activity decreased ($P < 0.05$) while urea concentration increased ($P < 0.05$), as measured ten months later.

The blood glucose level of captured hinds was higher ($P < 0.01$) than that of farmed ones, both at the time of capture and subsequently, at the time of taking the blood sample without the use of crush.

The expected consistent correlations between stress susceptibility related parameters ($r > 0.60$, $P < 0.05$) were found between CK activity and glucose level, CK activity and serum cortisol concentration, and glucose level and serum cortisol concentration in the farmed group treated with the probiotic preparation. Changes indicating a similar trend were observed in farmed groups at the first sampling in January as well.

Accident-free collection of blood samples from farmed deer required the use of crush. At the time of the first examination, resisting animals could not be distinguished from the "easily manageable" hinds by their blood plasma CK activities (93, 88 and 106 U/L, respectively) or serum cortisol concentrations (13, 180 and 119 nmol/L, respectively). With the advancement of gestation, CK activity decreased at a similar rate in farmed hinds.

The activity of ALT decreased in the treated farmed group ($P < 0.001$) after probiotic feeding. At the second blood sampling, significant differences ($P < 0.05$)

were found between the two groups, although the values were within the physiological limits.

Changes in the concentrations of glucose, urea and TP followed similar trends in the two groups. In the treated group, blood urea concentration rose between the two examinations ($P < 0.01$). Haemoglobin concentration decreased in both groups before the grazing season. This change was significant in the treated group.

Compared to the mean values found in samples taken in the winter period, the ratios of blastogenic transformation of lymphocytes following stimulation with the nonspecific mitogens PHA and ConA were more favourable in the early spring season. PHA and ConA values correlated in the control farmed group ($r = 0.62$). In this group the LST percentages elicited by PHA and ConA were negatively correlated with CK activity and glucose concentration (PHA – CK: $r = -0.30$; PHA – glucose: $r = -0.42$; ConA – CK: $r = -0.51$; ConA – glucose: $r = -0.46$). The LST % increase induced by PHA was 1.5 times higher in hinds of the treated group than in the control animals (Table 1).

Discussion

The blood parameters obtained in this study for red deer are consistent with the values reported in the literature (Wilson and Pauli, 1982*b*; Reid and Towers, 1985; Cross et al., 1988).

Studying the seasonal changes of blood constituents in normal farmed deer, Reid and Towers (1985) found high individual variation, especially in the CK activity of one-year-old fawns. The higher CK and LDH activities found at the time of capture in this study can be attributed to the muscle injury sustained by the animals while wriggling in the net. The high correlation ($r = 0.93$) demonstrated between the activities of CK and LDH enabled us to assess the extent of muscle cell membrane damage by determining the activity of only one of these enzymes, namely CK.

Differences in CK activity and serum cortisol concentration between hinds that resisted being put into a crush and "easily manageable" hinds were found only if the animals' aggressive behaviour led to soft tissue injury. The lower CK activity measured in early spring could be attributed to seasonal differences, the later stage of gestation, and perhaps also to the fact that at the time of sampling there were no weather front effects to which this wild species is particularly susceptible.

According to McAllum (1985), in stress-induced myodegeneration of deer the released myoglobin and the haemoglobin arising from haemolysis damage the renal tubules, resulting in a pathological increase in blood urea concentration

Table 1

Blood parameters of captured and farmed red deer hinds (mean ± standard deviation)

Blood parameters	Captured hinds		Farmed hinds[+++]			
	At capture[+] (n = 10)	10 months later[++] (n = 11)	Before treatment		75 days after treatment	
			Treated group (n = 12)	Control group (n = 12)	Treated group (n = 12)	Control group (n = 12)
CK[*] (U/L)	345 ± 319[a]	137 ± 33.3[b]	95 ± 38.6[b]	146.4 ± 62.5[b]	55 ± 9.9[b]	72 ± 19.0[b]
ALT[*] (U/L)	28.9 ± 6.1[ab]	32.1 ± 7.46[a]	35.2 ± 6.62[a]	36.2 ± 6.00[a]	24.7 ± 6.25[b]	32.5 ± 8.02[a]
LDH[*] (U/L)	427 ± 187[a]	NT	NT	NT	326 ± 66.1[a]	366 ± 78.8[a]
Glucose[*] (mmol/L)	12.35 ± 2.46[a]	11.84 ± 2.55[a]	7.78 ± 2.82[b]	9.83 ± 1.57[b]	8.38 ± 2.82[b]	8.66 ± 1.72[b]
Urea[*] (mmol/L)	5.73 ± 2.20[c]	11.94 ± 1.07[a]	6.00 ± 1.81[c]	7.33 ± 1.12[bc]	8.14 ± 1.62[b]	7.93 ± 0.88[b]
TP[*] (g/L)	67.4 ± 13.19[a]	61.6 ± 6.80[a]	63.3 ± 8.60[a]	67.8 ± 9.27[a]	62.7 ± 3,54[a]	63.4 ± 3.82[a]
Cortisol[**] (nmol/L)	92 ± 53.4[a]	115 ± 44.2[a]	123 ± 64.56[a]	137 ± 38.8[a]	157 ± 66.9[a]	147 ± 53.4[a]
PHA[***] (LST%)	NT	13.38 ± 7.60[a]	16.75 ± 4.89[a]	13.2 ± 5.12[a]	31.73 ± 17.54[b]	22.64 ± 13.42[b]
Con A[***] (LST%)	NT	13.80 ± 6.06[b]	12.09 ± 4.48[b]	11.04 ± 9.25[b]	19.58 ± 12.97[a]	18.56 ± 12.05[ab]
Hb[***] (g/100 mL)	NT	15.8 ± 4.60[c]	24.2 ± 5.09[a]	21.2 ± 3.2[b]	17.4 ± 3.53[cb]	17.4 ± 3.11[cb]

[a,b,c]Values marked with different superscripts within a row are significantly different ($P < 0.05$); NT = not tested;
[+]Immobilisation by xylazine-ketamin; [++]Physical restraint without crush; [+++]Restraint with crush;
[*]from plasma; [**]from serum; [***]from total blood

(>12.00 mmol/L). The high urea concentration (12 mmol/L) measured in the captured group at the second blood sampling, as compared to that found at capture (5.7 mmol/L), can probably be attributed to the effect of artificial environment and feeding.

Blood samples from captured hinds could be taken without the use of crush, as in the semi-dark environment of the narrow treatment frame the animals reacted to the presence of humans by myofibrillar tremors and stiffening. According to studies conducted by Dantzer and Mormede (1983) in domestic animals, emotional stimuli elicit the alarm reaction stage of the general adaptation syndrome. The elevated blood glucose level (~12.00 mmol/L) of captured red deer may be associated with a pronounced sympathicomimetic effect and enhanced adrenaline release at the time of blood sampling. These high blood glucose levels indicate that such animals are more sensitive even to moderate external stimuli than farmed deer accustomed to being placed in crush.

In a previous work (Zomborszky et al., 1993), captured red deer hinds were found to have 4 times as high serum cortisol concentration as hinds reared under artificial management conditions, when the crush technique was used for blood sampling. The mean serum cortisol concentration of yearling fawns derived from captured hinds (~127 nmol/L) was about half the value measured in their dams. In this study, no major differences were found in the mean serum cortisol concentrations of blood samples taken from hinds immobilised at capture, those taken without the use of crush, or collected from farmed animals restrained in crush. The high values of standard deviation suggest the importance of individual differences depending on the momentary status.

Hanlon et al. (1995) reported that stress-inducing ACTH treatment did not affect the lymphocyte response to ConA stimulation in feral and "mixed" deer. In this study, no differences were demonstrated between captured and farmed hinds either in the ratio of lymphocyte stimulation induced by PHA and ConA in blood samples collected in the winter period. In early spring, the ratios of lymphocyte transformation induced by nonspecific mitogens were more favourable.

In red deer, the most severe risk posed by stress susceptibility induced immunodeficiency is that it facilitates the spread of infection within the herd if *Mycobacterium bovis* is present (Zomborszky et al., 1995). When pigs were fed a preparation of similar composition as the probiotic applied in this study, the incidence of stress-induced PSE meat and CK activity at slaughter decreased (Zomborszky-Kovács, 1994). Besides exerting a hepatoprotective effect, shown by the decreased ALT levels, Ascogen® elevated the blood urea level of red deer, but the change was within the physiological limits. The lower mean activity of the myogenic enzymes CK and LDH in the treated animals indicates that the probiotic has a protective effect in stress-induced damages. Glawisching and Pedit (1992)

reported that Ascogen® has an immunomodulating effect in pigs. The favourable change observed in PHA-induced lymphocyte stimulation percentage in the present study shows that deer belonging to the treated group had better humoral B-cell-mediated immune responsiveness than the control hinds.

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EFFECT OF LIPID PEROXIDE LOADING ON LIPID PEROXIDATION AND ON THE GLUTATHIONE AND CYTOCHROME SYSTEMS IN RABBITS

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New Zealand White rabbits (6 males and 6 females) were fed a diet of high lipid peroxide content (peroxide value: 249.05 meq/kg fat) for 21 days. Twelve rabbits served as controls (peroxide value: 40.3 meq/kg fat). The lipid peroxide loading did not cause clinical signs. The rate of lipid peroxidation, as measured on the basis of thiobarbituric acid reactive substances (TBARS), was significantly ($P < 0.05$) higher in all of the investigated tissues, in the following order: liver > red blood cells (RBC) > blood plasma. Reduced and oxidised glutathione content was higher in the blood plasma ($P < 0.01$) and liver ($P < 0.001$) of rabbits exposed to the peroxide load. Lipid peroxide loading decreased the activity of glutathione peroxidase in the blood plasma, RBC haemolysate and liver and that of glutathione reductase in the liver. The amount of cytochrome P450 (both CO- and metyrapone-reduced) and the activity of cytochrome c (P450) oxidoreductase in the microsomal fraction of the liver homogenate were also lower in the group exposed to lipid peroxide load. Subchronic alimentary lipid peroxide loading in the presence of sufficiently high levels of antioxidants in the complete feed was found to increase the rate of lipid peroxidation and markedly lower the activities of both the glutathione and xenobiotic transforming enzyme systems without causing any clinical signs of toxicity.

Key words: Malondialdehyde, glutathione, cytochromes, lipid peroxide, rabbit

Subchronic alimentary lipid peroxide loading initiates peroxidative processes in many tissues, especially in the liver, as was found in guinea pigs and mice (Miyazawa et al., 1986). It has also been observed that the liver of rabbits fed different high-fat diets is very susceptible to oxidative stress (Slim et al., 1995).

There are several theories on the intestinal absorption of lipid hydroperoxides. Some authors suggest that hydroperoxides are not absorbed from the small intestine after alimentary lipid peroxide intake (Glavind et al., 1971). Others have

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shown that chylomicrons within the lymphatic system have primary importance in the transport of alimentary lipid peroxides (Aw et al., 1992) taken up by the liver (Kanazawa et al., 1985). Oxidised lipid metabolites formed after the consumption of thermally oxidised oils were found to increase the amount of thiobarbituric acid reactive substances (TBARS) in the blood plasma of human subjects (Naruszewicz et al., 1987) and chickens (Sheehy et al., 1994).

The stable redox state of cells is very important for the essential vital functions. Among other thiols, glutathione plays an important role in the maintenance of cellular redox status and antioxidant defence (Meister and Anderson, 1983). Regularly, there is enhanced oxidation of reduced glutathione (GSH) to the oxidised form (GSSG) due to the increased production or presence of reactive oxygen substances. For that reason, the amount of GSSG has been proposed for use as an index of oxidative stress of tissues (Verbundt et al., 1995). The recycling of GSSG to GSH is limited because it depends on either glutathione reductase activity, NADPH supply, or both (Ji, 1995). Accumulation of GSSG is toxic to the cell because it may increase disulphide cross-linkage of proteins (Ji et al., 1988). The liver has a central role in this process because 90% of the circulating GSH is synthesised in the liver (Lu et al., 1990).

The cytotoxic effects of lipid peroxidative processes are partly based on the effect of reactive aldehydes, particularly alkenals generated during those processes. The toxic metabolites primarily damage the biological membranes and the membrane-bound enzymes, e.g. members of the cytochrome P450 family (Bestervelt et al., 1995; Proulx and du Souich, 1995).

The effect exerted by diets of high lipid peroxide content on lipid peroxidation has been investigated in numerous species; however, before this study no data were available on the effect of lipid peroxide loading on the cytochrome system in rabbits. The objective of the present study was to investigate the effect of subchronic alimentary lipid peroxide loading on the rate of lipid peroxidation as well as on the activities of the glutathione and xenobiotic transforming enzyme systems in rabbits. The rancidity of fats is a common problem in the feed industry, and many data are available on its negative effects on the health status and production traits of animals (Andrews et al., 1960; Fuhrmann et al., 1996; Hara et al., 1996).

Materials and methods

Twelve New Zealand White rabbits in both the treated and the control group (6 females and 6 males in each group) were used for the study. The males and females were littermates in each of the two groups. At the start of the trial the age of the animals was 79 days and their average body weight was 2.48 ± 0.21 kg.

The animals were kept in individual cages during the experiment which lasted 21 days. Free access to feed and drinking water was provided.

The peroxide content of the experimental diet was increased by adding ground maize containing oxidised sunflower oil at a level of 2% (w/w). The ground maize was treated with 50% (w/w) crude sunflower oil and the mixture was incubated at 60 °C for 24 h with continuous air supply.

The nutrient content of the feed was determined according to methods corresponding to the Hungarian National Standard (Hungarian Feed Codex, 1988). The peroxide and acid values were measured according to Chandler (1993). The peroxide value of the dietary fat was determined after cold extraction of lipids with n-hexane, adding potassium iodide, and free iodine was determined by titration of sodium thiosulphate in the presence of starch indicator. The acid value of the dietary fat was also determined after cold extraction with n-hexane and the free fatty acids were measured by titration of potassium hydroxide using phenolphthalein as indicator. The nutrient content of feeds is shown in Table 1.

Table 1

Nutrient content of feeds

Nutrient content	Control diet	Experimental diet
Dry matter (%)	90.50	90.80
Ash (%)	8.90	8.10
Crude protein (%)	19.97	19.33
Crude fat (%)	3.40	3.70
Crude fibre (%)	14.96	12.14
Nitrogen-free extract (%)	43.91	47.35
Acid value (meq /kg fat)	12.30	99.20
Peroxide value (meq/kg fat)	40.30	249.05

The animals were killed by cervical dislocation in light narcosis and samples for the determination of lipid peroxide formation and parameters of the glutathione redox system in the blood plasma, red blood cell (RBC) haemolysate (1:9 in redistilled water), and liver (1:9 homogenate in physiological saline and its 10,000 *g* supernatant fraction) were taken at the end of the investigation. The parameters of the cytochrome system were determined in the microsomal (10,000 *g* supernatant) fraction of liver homogenate prepared by gradient centrifugation (Cinti et al., 1972).

The malondialdehyde content of samples was measured by the 2-thiobarbituric acid reaction (Placer et al., 1966). The amount of reduced glutathione (GSH) was determined by a photometric method (Sedlak and Lindsay,

1968) and that of oxidised glutathione (GSSG) in the form of GSH after recyclisation with glutathione reductase in the presence of NADPH with GSH subtraction (Tietze, 1969). The activity of glutathione peroxidase was measured by an end-point direct assay using GSH and cumene-hydroperoxide co-substrates, and the GSH loss was measured by photometric assay (Matkovics et al., 1988). Glutathione peroxidase activity was expressed in units: one unit meant 1 nmol GSH oxidation min^{-1} at 25 °C. The activity of glutathione reductase was followed by measurement of the oxidation of NADPH in the presence of GSSG (Matkovics et al., 1988). Glutathione reductase activity was expressed in units: one unit meant 1 μmol NADPH oxidation min^{-1} at 37 °C.

The protein content of blood plasma and RBC haemolysate was determined by the biuret method (Weichselbaum, 1946) and using the Folin phenol reagent for the 10,000 *g* supernatant fraction of liver homogenate (Lowry et al., 1951) because of the moderate turbidity of the homogenate samples.

Quantitative analysis of cytochrome P-450 was carried out in the form of P-450:CO (Omura and Sato, 1964) and P-450:metyrapone (Mitani et al., 1982) complexes. The activity of cytochrome c (P-450) oxidoreductase was measured by the method of Williams and Kamin (1962).

The differences between means were calculated by paired Student's *t*-test (Snedecor and Cochran, 1976).

Results

Malondialdehyde (TBARS) content was significantly higher in the blood (blood plasma and RBC haemolysate) and liver homogenate of treated rabbits than in the controls (Table 2), indicating that lipid peroxide loading initiated free radical formation in the blood and liver of animals.

Reduced glutathione content was significantly higher in the blood plasma and liver homogenate in the treated rabbits than in the controls but remained unchanged in the RBC haemolysate, which was a surprising finding in view of the lipid peroxide load. Oxidised glutathione content was significantly higher in the blood plasma, RBC haemolysate and liver homogenate of the treated rabbits (Table 2), which elevation is a well-known consequence of free radical generated processes in biological systems.

Glutathione peroxidase activity was significantly lower in the blood plasma, RBC haemolysate and liver homogenate while glutathione reductase activity only in the liver homogenate of the treated animals (Table 2), suggesting dissimilar sensitivity of the enzymes to the same effect and partly explaining the changes of reduced and oxidised glutathione in the same compartment.

Table 2

Effect of subchronic alimentary lipid peroxide loading on the lipid peroxide and antioxidant status of blood plasma, RBC haemolysate and liver homogenate (mean ± S. D.)

Parameter	Blood plasma		RBC haemolysate		Liver homogenate (10,000 *g* supernatant)	
	Control	Treated	Control	Treated	Control	Treated
MDA (mmol/L, mmol/g wet wt.)	2.89 ± 0.72	3.73 ± 0.72^{x}	3.25 ± 0.39	3.82 ± 0.51^{x}	3.61 ± 0.82	4.96 ± 0.68^{x}
GSH (mmol/L, mmol/g wet wt.)	0.08 ± 0.01	0.12 ± 0.01^{xx}	1.05 ± 0.08	1.02 ± 0.07	1.36 ± 0.47	3.57 ± 1.03^{xxx}
GSSG (µmol/L, mmol/g wet wt.)	0.12 ± 0.08	1.01 ± 0.38^{xxx}	4.28 ± 1.83	9.01 ± 2.53^{xx}	5.24 ± 3.09	62.23 ± 20.69^{xxx}
GSH-Px U/g 10,000 *g* protein	2.73 ± 0.52	1.51 ± 0.22^{xx}	2.64 ± 0.72	1.87 ± 0.49^{xx}	86.29 ± 27.46	26.81 ± 3.03^{xxx}
GSSG-R U/g 10,000 *g* protein	9.62 ± 2.97	7.48 ± 2.28	11.36 ± 5.78	11.41 ± 3.90	133.06 ± 25.48	45.91 ± 24.64^{xxx}

Levels of significance: $^{x}P < 0.05$; $^{xx}P < 0.01$; $^{xxx}P < 0.001$

The amount and/or activity of the cytochrome system in the microsomal fraction of the liver was significantly lower in the treated rabbits than in the controls as a result of subchronic alimentary lipid peroxide loading (Table 3).

Table 3

Effect of subchronic alimentary lipid peroxide loading on the parameters of the microsomal xenobiotic transforming enzyme system of the liver (mean ± S. D.)

Parameter	Control	Peroxide-treated
Cytochrome P450 (CO-red., mmol/g)	9.76 ± 1.02	0.90 ± 0.16^{xxx}
Cytochrome P450 (metyrapone-red., mmol/g)	1.55 ± 0.19	0.95 ± 0.16^{xx}
Cytochrome c (P450) oxidoreductase (mmol/g/min)	1262 ± 141	806 ± 116^{xxx}

Levels of significance: $^{xx}P < 0.01$; $^{xxx}P < 0.001$

Discussion

Subchronic alimentary lipid peroxide loading caused lipid peroxidation, which was supported by the significantly higher malondialdehyde content in all of the investigated compartments. That effect is well known from previous reports on rodents such as guinea pigs and mice (Miyazawa et al., 1986). The susceptibility of rabbit liver to peroxidative processes is also known from earlier reports (Mézes et al., 1994; Slim et al., 1995).

The higher concentration of oxidised glutathione in the blood and liver of rabbits subjected to lipid peroxide load was due to enhanced lipid peroxide formation, which effect was also found in a rat heart model system (Ishikawa and Sies, 1984). The real mechanism of the higher GSH found in this study cannot be fully explained and requires further investigations.

Lipid peroxidative processes primarily damage the biological membranes and decrease the amount of cytochrome P450 and the activity of cytochrome c oxidoreductase in liver microsomal preparations of rats (Bestervelt et al., 1995) and rabbits (Proulx and du Souich, 1995). The decreased amount of cytochrome P450 and the reduced activity of cytochrome c oxidoreductase as found in the present study can be explained by the above-mentioned findings, because lipid peroxide formation was indicated by the elevated malondialdehyde level of the liver.

In summary, subchronic alimentary lipid peroxide loading caused an increase in lipid peroxidation in the blood and liver, suggesting that lipid peroxidation products were transported and only partially decomposed at the site of absorption. The amount and/or activity of the glutathione redox system decreased as a result of lipid peroxide loading. The absence of clinical signs of toxicity shows that the glutathione redox enzymes effectively eliminated most of the reactive oxygen metabolites. The results of this study underline the need to pay attention to the effects of diets containing high levels of lipid peroxide irrespective of whether or not clinical signs of toxicity are present, since the activities of the glutathione and cytochrome systems could become impaired. Owing to sufficient antioxidant (vitamins A and E, selenium) supplementation of the complete feed, the diet of high lipid peroxide content did not lead to adverse clinical consequences in this experiment; however, in view of the impaired defence mechanism, contamination of the feed or drinking water with any other toxic substance could have caused irreversible damage.

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SUSPECTED ADVERSE DRUG REACTIONS IN THE HUNGARIAN VETERINARY PRACTICE BETWEEN 1982 AND 1992

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The most characteristic adverse drug reactions observed after the use of home-manufactured or imported veterinary medicinal products in Hungary between 1982 and 1992 included toxicosis, severe local reaction, lack of efficacy, and presence of residues in the edible tissues of food animals. The causes of adverse drug reactions comprised manufacturing defects, lack of chemical or microbiological stability, misuse or extra label use, and neglecting the warnings in the directions for use. Collection and analysis of data relating to adverse drug reactions are indispensable for the prevention of similar cases. The authorities can facilitate data collection by supplying veterinary practitioners with the necessary report forms.

Key words: Adverse reactions, drug, toxic effect

The official quality control of veterinary medicinal products has been performed in Hungary by the State Control Institute for Veterinary Biologicals, Drugs and Feeds since 1982. Since that time, quality claims related to drugs and adverse reactions observed after drug use have been reported to that institute. The present paper deals only with cases belonging to the latter category, i.e. adverse drug reactions. It gives account of all active ingredients which have been associated with some abnormality observed and reported. In the majority of cases specialists of the institute, in co-operation with the competent county veterinary station, carried out on-site investigations as well. Where it has been revealed by the investigations, the cause of adverse drug reactions is also given. However, the detailed description of individual cases is beyond the scope of this paper.

In the period between 1982 and 1992, adverse drug reactions were reported after the use of veterinary medicinal products containing 15 different active ingre-

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dients or active ingredient combinations. The preparations included both home-produced and imported ones.

The cases, grouped according to the type of the adverse drug reaction, are presented in this paper.

Toxic effect

In the period between 1983 and 1991, suckling piglet mortality was reported several times after the administration of iron-dextran injectable preparations manufactured by different companies. Süveges and Glávits (1974) suggested that the observed iron toxicity might have been due to the individual hypersensitivity and vitamin E deficient status of the piglets. In our opinion, the low free iron content of parenterally administered iron preparations is also an important factor in product safety. This value should not exceed 0.2 mg/ml even at the end of the expiration period. Therefore, free iron content and its changes during storage are very important considerations when evaluating the registration documents of iron preparations.

Neglecting of the manufacturing regulations led to poisoning in the case of two preparations. During the manufacture of the injection containing sodium selenite as active ingredient, incomplete dissolution of the powdered active ingredient resulted in inhomogeneity of the batch. Therefore, vials filled early in the dosing process had higher selenium concentration than specified. Random sampling carried out during the internal quality control failed to detect this phenomenon. One of these vials was administered to 2- to 4-week-old lambs in a sheep flock. After the injection, some of the animals died of selenium toxicosis. Unused vials sent for claim by the attending veterinarian contained large amounts of active ingredient, sometimes as much as four times the declared value. After the intramuscular administration of sodium selenite to sheep, Blodgett and Bevill (1987) found that the LD_{50} of selenium was 0.7 mg/kg body weight, while in a similar study Caravaggi et al. (1970) obtained a value of 0.455 mg/kg. Muth and Binns (1964) reported that a single subcutaneous dose of 0.66 mg/kg selenium administered as sodium selenite in aqueous solution resulted in the death of all the 12 ewes treated. According to our calculations, in the case reported by us the animals must have received 2.5–3 times the LD_{50} value as a subcutaneous injection. The batch control samples taken during the manufacturing process and later samplings from the same batch met the specifications. After the adverse drug reaction had been reported, the batch was sequestered and, after analysis, withdrawn.

As a result of a so-called "cross-contamination", *narasin* was accidentally mixed into a commercial dog food. This case can also be considered a technological failure. The feed mill produced premixes and finished feed on the same equip-

ment and failed to establish a compulsory sequence for the different products it manufactured. Therefore, it could happen that after a narasin-containing broiler chicken premix a commercial dog food was manufactured, without a preliminary flushing of the mixing equipment with neutral carrier. The dog food, which contained 69–1,065 mg narasin per kg of food, resulted in the death of dogs consuming it. Papp et al. (1989) established that in fatal cases the dogs took up narasin in a dose of 1.2–19 mg/kg body weight. In other cases, similar doses caused only more or less severe clinical signs of toxicity, indicating that the sensitivity of individual dogs varies widely. Based upon data of the literature, the oral LD_{50} of narasin was reported to be 10 mg/kg body weight. Following a measure taken by the authorities, the narasin-contaminated dog food was withdrawn.

Once the extra label use of a *lincomycin* and *spectinomycin* containing powder resulted in the death of rabbits treated with the preparation.

In another case an injectable preparation containing ivermectin was used beyond the scope of the indications in the data sheet, and it led to the death of fox-cubs. Taking into account the usual dosage of ivermectin, the cubs weighing approximately 1 kg received an about tenfold overdose. A similar case was reported by Keck (1994) after the subcutaneous administration of a non-licensed ivermectin preparation to dogs.

The intravenous administration of an injection containing *drotaverin* as active ingredient to a 18-year-old horse suffering from colicky heart failure resulted in the animal's death. The attending veterinarian took no notice of the contraindication mentioned in the directions for use, i.e. that the preparation should not be administered to animals with heart failure and impaired renal or liver function.

Systemic reaction or severe local reaction

On one occasion, allergic reaction and death were observed after the simultaneous treatment of 1- to 3-week-old calves with injections containing *vitamin D_3* and *streptomycin-penicillin* as active ingredient.

Ten out of 307 sheep treated with an injection containing *diethylcarbamazine* as active ingredient died of gas gangrene. The preparation was sterile; thus, the adverse drug reaction had no causal relation with the product administered.

In one instance, the administration of an injection containing *iron-dextran* as active ingredient caused a severe local reaction in piglets. The tests revealed that the preparation was contaminated with saprophytic bacteria. The batch was withdrawn.

In a cow herd, an alkaline detergent used for cleaning milking machines accidentally got into a *teat dip preparation*. The udders and teats of treated cows became ulcerated and the herd had to be emergency slaughtered.

After feeding pigs with a granulated preparation containing *mebendazole* as active ingredient, vomiting, spasms, and diarrhoea occurred. The incriminated preparation was fed to pigs under experimental conditions at the usual dose. The results of the experiment did not reveal any relationship between administration of the drug and the clinical signs observed. According to Marsboom (1973), mebendazole administered at a dose of 10 or 20 mg/kg body weight caused softer faeces or diarrhoea in some of the treated pigs without any other symptoms. In his opinion, the development of clinical signs depended on the individual sensitivity of animals.

Lack of efficacy

Lack of efficacy and mortality of honey bees caused by the mite *Varroa jacobsoni* were observed after the use of fumigating strips containing *amitraz* as active ingredient. According to the analytical tests, decomposition of the active ingredient had taken place because of the high moisture content of the preparation. Further investigations revealed that the manufacturing technology of the product had been changed without thorough studies and prior approval by the authorities. As a result, imperfectly dried strips were placed into impermeable packaging. The product has been cancelled from the registry.

Lack of efficacy was reported after the use of an injection containing *chorionic gonadotropin* and *oestradiol benzoate* as active ingredient in pigs; however, this could not be confirmed by subsequent investigations.

Residues

During the period of data collection, residues were detected in the edible tissues of pigs for slaughter on two occasions. In one case the residue was identified as *sulfamethazine*. In the other case *monensin* was identified. In the latter case, the presence of residue was probably due to the feeding of a poultry diet.

Discussion

The reporting of adverse drug reactions has been rendered compulsory and regulated in Hungary by a decree already since 1981. In addition, Annex no. 5 of the Ministry of Agriculture Decree no. 22/1996 (VII.9.) FM currently in force

contains a report form which gives information on the format and content of notifications.

The collection and analysis of data on adverse drug reactions have recently become the centre of interest. Since 1 January 1995, a veterinary pharmacovigilance system implemented on the basis of the relevant regulations has been operating in the European Union. This system has been described in detail by Boisseau (1994). The collection of data on adverse drug reactions obviously helps us in the assessment of risks associated with the use of active ingredients and preparations. This is especially important if new active ingredients are to be used.

Elucidation of adverse drug reactions due to technological faults of the manufacturing process facilitates the prevention of similar cases. The accumulated experience contributes to the reliable assessment of registration documents and the periodic review of marketing authorisations. The relevant Hungarian requirements have been described by Bernáth (1995).

Veterinary practice is a source of important data on adverse drug interactions, too. Reilly and Isaacs (1983) point out that the use of multiple drug therapy, which is often necessary in veterinary practice, may result in drug interactions. In their opinion, the incidence of undesirable drug interactions can best be minimised by knowing the principles which underlie the mechanisms by which they occur. Naturally, it is also very important that the directions for use should include warnings to potential drug interactions.

Based upon data of the literature, Gray (1994) stated that in human practices only 6 per cent of suspected adverse reactions were reported. It is very likely that only a low percentage of cases is reported in the veterinary field, too. This assumption is supported by Ibrahim et al. (1994) who point out that in Germany the number of reported "adverse drug reactions" per year is very low, compared to the number of practising veterinarians in the country, the number of authorised drugs and the number of treated animals. According to the latter authors, most veterinarians make use of the pharmacovigilance system only when a fatal event occurs and possibly demands of indemnity are addressed to them by animal owners. This statement is also supported by the fact that in Germany 71% of the adverse reaction reports between 1986 and 1993 were made after deaths of animals.

According to Gray (1994), the number of reports are influenced by the following eight factors: (1) Severity of the reaction. (2) Duration of marketing. (3) Attribution of blame by the animal owner. (4) The reaction has previously been seen by the veterinary surgeon or is well known to the profession from the literature. (5) Legal implications. (6) The unusual or unique nature of the event. (7) National regulations on the notification of suspected adverse reactions. (8) Media coverage.

It is obvious that all the above factors are of decisive importance; however, because of the complexity of the problem there is no complete list of the influencing factors. In our opinion, the number and ratio of reported cases are markedly affected by the technical conditions as well as the time and labour demand of notification. The veterinary authorities can perhaps support the collection of important data most effectively if they supply veterinary practitioners with detailed questionnaires and forms that facilitate the reporting of adverse drug reactions. The 24-hour reception of data by telematic means (phone, fax, e-mail) at the competent authorities must also be ensured.

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RAPID FREEZING OF MOUSE EMBRYOS IN ETHYLENE GLYCOL AT DIFFERENT PREIMPLANTATION STAGES

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The objectives of this study were to examine the effects of a rapid freezing protocol on the survival and *in vitro* development of mouse embryos cryopreserved in ethylene glycol (EG) at different preimplantation stages, and secondly, to investigate the effect of exposure to 3.0 M EG with 0.25 M sucrose on the survival and *in vitro* development of mouse embryos without freezing at different developmental stages. To perform the rapid freezing procedure, embryos were equilibrated in Dulbecco's phosphate buffered saline (DPBS) containing 3.0 M EG and 0.25 M sucrose (freeze medium) for 20 min and loaded into 250 µl straws in a single column of freeze medium. The straws were held in liquid nitrogen (LN_2) vapour for 2 min and immersed into LN_2. Embryos were thawed in a 37 °C water bath for 20 sec and transferred to DPBS supplemented with 0.5 M sucrose (rehydration medium) for 10 min and cultured for 24 to 96 h in HTF (Human Tubal Fluid) plus 4 mg/ml BSA (Bovine Serum Albumin). Significant differences were found in the survival and development of mouse embryos at different developmental stages rapid frozen in EG and sucrose: two cell 43/84 (51%), 4–8 cell 44/94 (47%), morula and early blastocyst 56/70 (80%), expanding and expanded blastocysts 10/59 (17%) ($p < 0.05$). These data indicate that the developmental stage in which mouse embryos are subjected to this quick freeze protocol affects survival and development *in vitro* and the majority (80%) of morula and early blastocyst stage embryos survive the procedure. No significant differences were observed in the *in vitro* developmental capacity of embryos at different developmental stages after treatment with high concentrations (3.0 M) of EG solution without freezing. Further investigations are underway to better understand the reasons for different survival rates of embryos frozen at different developmental stages using the present procedure.

Key words: Mouse embryo, embryo freezing, rapid freezing, ethylene glycol

The first report of live-born young from cryopreserved mouse embryos was published in 1972 (Whittingham et al., 1972). Since that time similar success has

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been achieved for several mammalian species and it has been proven repeatedly that neither practical embryo transfer (ET), nor basic research in reproduction can exist without frozen embryos cryopreserved at different developmental stages. Excellent general reviews are available on basic and applied aspects of the cryobiology of mammalian embryos (Leibo, 1986; Rall, 1987, Niemann, 1991). Many different procedures have been developed during the past 25 years resulting in successful cryopreservation of embryos. These procedures can be divided into three groups: (1) controlled slow freezing, (2) rapid/ultrarapid freezing, and (3) vitrification. Each approach results in a characteristic sequence of changes in the osmotic volume of cells during the steps of cryopreservation (Mazur, 1990).

Rapid freezing procedures provide an alternative method for eliminating controlled slow freezing during embryo cryopreservation. In this approach embryos are dehydrated prior to cooling in moderately concentrated solutions of cryoprotectants prior to rapid cooling to –196 °C. High levels of survival have been reported for mouse embryos equilibrated in a wide range of mixtures of sucrose or lactose plus either glycerol, DMSO or ethylene glycol (Takeda et al., 1984; Szell and Shelton, 1986; Szell and Shelton, 1987; Trounson et al., 1987; Abas-Mazni et al., 1989; Abas-Mazni et al., 1990; Takahashi and Kanagawa, 1990; Rayos et al., 1992*a*, 1992*b*; Rayos et al., 1994).

This study was conducted to examine the effects of a rapid freezing protocol (direct plunging into liquid nitrogen vapour) on the survival and *in vitro* development of mouse embryos cryopreserved in ethylene glycol at different preimplantation stages. Besides freezing the embryo toxicity of the ethylene glycol solution was also studied on mouse embryos at different preimplantation stages. Our aim was to evaluate the efficiency of the freezing/thawing technology on the survival of embryos frozen at different preimplantation stages. Until now the cooling procedure described below was used only for cryopreservation of mouse oocytes, one cell stage and early cleavage stage mouse embryos (4, 8, 16 cell and morulae) (Abas-Mazni et al., 1990; Rayos et al., 1992*a*, 1992*b*; Rayos et al., 1994). This technology has not been reported for freezing compact morulae and different stages of blastocyst (early, expanding and expanded) mouse embryos.

Materials and methods

Embryo production

Six to eight week old CB6F1 female mice were selected and subjected to the following hormonal treatment schedule: First 10 IU PMSG (Sigma) was administered ip. and 46–48 h later the animals were treated ip. with 10 IU hCG (Sigma) in order to support the ovulation of matured follicles. One cell embryos were har-

vested 20–23 h after pairing with males of the same strain and hCG injection. Modified human tubal fluid (MHTF/HEPES buffered HTF/, Irvine Scientific, Irvine, CA, USA) supplemented with 4 mg/ml BSA (Bovine Serum Albumin, Sigma) was used to collect the embryos. In a following step the cumulus cells were removed from the surface of the embryos by treatment with hyaluronidase [MHTF + 4 mg/ml BSA medium supplemented with 300 μg/ml hyaluronidase (Hyaluronidase, Sigma)]. The embryos were rinsed three times in MHTF containing 4 mg/ml BSA medium before they were transferred into the culture medium.

Embryo culture

The embryo cultures were performed in human tubal fluid [Human Tubal Fluid (HTF), Irvine Scientific, Irvine, CA, U.S.A.] medium containing 4 mg/ml BSA, under mineral oil at 37 °C in 5% CO_2 plus 95% room air at maximal humidity. NUNC dishes (Multidish 4, Nunclon) containing 400 μl culture medium covered with 400 μl equilibrated mineral oil (Mineral Oil, Sigma) were used for embryo culture and embryo development was monitored twice daily.

Embryo freezing

Twenty-four to 96 h after collection embryos were removed from culture and frozen at the following stages: 2 cell, 4–8 cell, compact morulae and early blastocyst, expanding and expanded blastocyst. First the embryos were rinsed three times in Dulbecco-PBS (Sigma) solution containing 10% FBS [Fetal Bovine Serum (FBS), Irvine Scientific, CA, U.S.A.]. To perform the quick-freeze procedure, embryos were equilibrated in Dulbecco's phosphate buffered saline (DPBS) + 10% FBS + 0.25 M sucrose (Sucrose, Sigma) + 3.0 M ethylene glycol (Ethylene Glycol, Sigma) /freeze medium/ for 20 min at room temperature (22–26 °C) and loaded in a single column of freeze medium into 250 μl straws (4–5 embryos per straw). After equilibration the straws containing the embryos were held in the LN_2 vapour for 2 min and quickly immersed in LN_2. The diagram of the rapid freezing procedure is shown in Fig. 1.

Thawing of frozen embryos

The embryos were thawed by gentle agitation in 37 °C water bath for 20 sec followed by transfer into DPBS + 10% FBS solution enriched with 0.5 M sucrose (rehydration medium) and kept for 10 min at room temperature. After rehydration the embryos were transferred and rinsed three times in fresh DPBS + 20% FBS, then three times in MHTF + 4 mg/ml BSA medium.

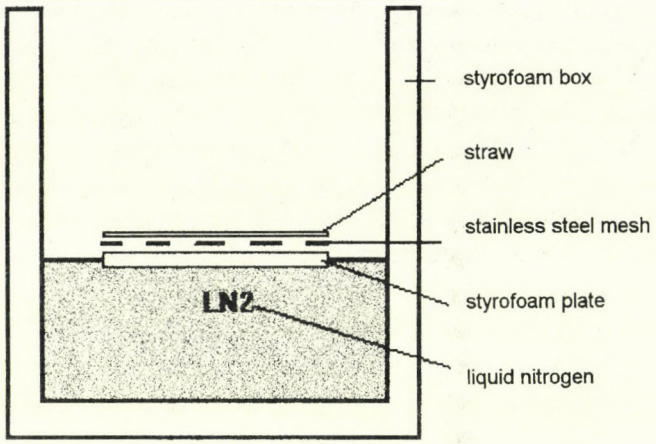


Fig. 1. Diagram of the rapid freezing procedure. The straws containing the embryos are placed on a styrofoam plate floating in the liquid nitrogen bath. The distance between the embryos and the surface of the liquid nitrogen is approximately 3–5 mm

Investigation of viability of frozen/thawed embryos

The embryos were cultured in HTF supplemented with 4 mg/ml BSA medium. The culture conditions were identical to that used before freezing (see above). The development of embryos was monitored twice daily. Survival of embryos was assessed by their ability to develop into expanded blastocysts during the subsequent culture period *in vitro*. Survival of blastocysts after thawing also required expansion or re-expansion of the blastocoel after several hours in culture.

Determination of toxicity of the freezing solution

The toxicity of freezing medium was investigated by treatment of embryos at different developmental stages, from 1 cell stage to expanding and expanded blastocysts. The embryos were kept in DPBS + 10% FBS containing 3.0 M EG and 0.25 M sucrose (freeze solution) for 20 min. They were then transferred into DPBS + 10% FBS containing 0.5 M sucrose (rehydration solution). After 10 min of rehydration the embryos were rinsed three times in DPBS + 20% FBS, then three times in MHTF + 4 mg/ml BSA medium. The embryos were cultured for 24–96 h in HTF + 4 mg/ml BSA solution and their development was checked twice a day. Survival of embryos was based on their ability to continue development upon subsequent culture *in vitro*. Survival of blastocysts required re-expansion of the blastocoel after several hours in culture. Data were analyzed for statistical significance using the chi-square test.

Results

Toxicological investigation

Table 1 and Fig. 2 show the effect of exposure to 3.0 M EG with 0.25 M sucrose on the survival of mouse embryos without freezing.

Table 1

The effect of exposure of embryos to 3.0 M ethylene glycol with 0.25 M sucrose on the survival of mouse embryos without freezing

Developmental stages	Embryo	
	Treated (n)	Developed *in vitro* (%)
1 cell	52	46 (88.46)
2 cells	52	44 (84.61)
4–8 cells	51	48 (94.11)
CM and EABL	53	52 (98.11)
EXBL and EDBL	50	48 (96.00)

CM = compact morulae; EABL = early blastocyst; EXBL = expanding blastocyst; EDBL = expanded blastocyst

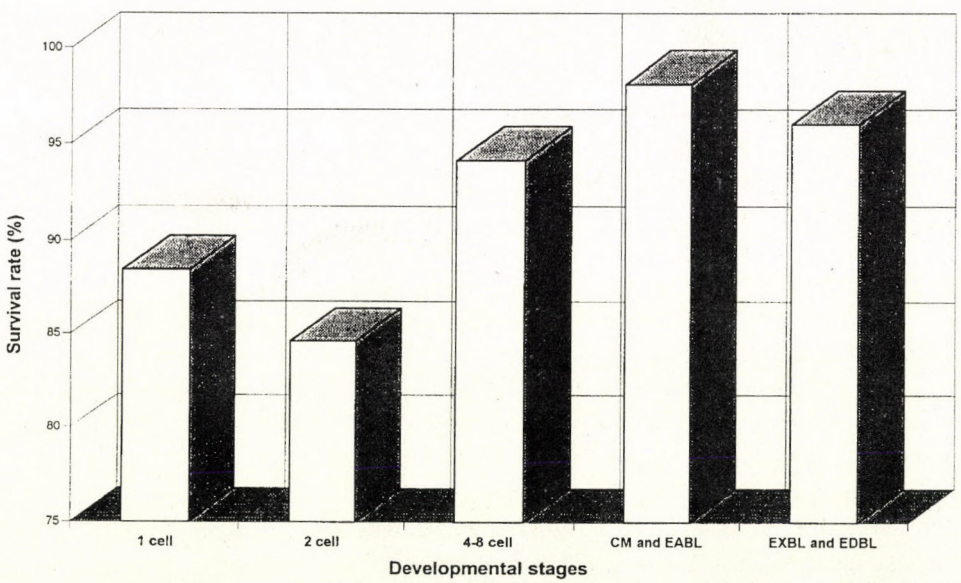


Fig. 2. The effect of exposure of embryos to 3.0 M ethylene glycol with 0.25 M sucrose on the survival of mouse embryos without freezing

Very high percentages (84.6–98.1%) of embryos exposed to the freezing medium for 20 min without freezing developed further when cultured *in vitro*. The *in vitro* development rates were similar to those of control embryos (37/40; 92%). Our results indicate that the 20-min 3.0 M EG treatment was not toxic to the embryos as determined by their development after treatment. No significant differences were found in the survival and development of mouse embryos at different developmental stages equilibrated in 3.0 M EG followed by rehydration in 0.5 M sucrose.

Embryo freezing

Results obtained after rapid freezing of mouse embryos at different developmental stages are presented in Table 2 and Fig. 3.

Table 2

The results of *in vitro* culture of thawed mouse embryos rapid frozen at different developmental stages

Embryo		Result	
Age	Frozen/Thawed (# of replicates)	Developed in culture n	Developed in culture %
2-cell	84 (6)	43	51[a]
4–8 cell	94 (5)	44	47[a]
CM and EABL	70 (5)	56	80[b]
EXBL and EDBL	59 (4)	10	17[c]

Values with different superscripts (a, b, c, d) are significantly different ($p < 0.05$). CM = morula; EABL = early blastocyst; EXBL = expanding blastocyst; EDBL = expanded blastocyst

Significant differences were found in the survival and development *in vitro* of mouse embryos at different developmental stages quick frozen in EG and sucrose. When early cleavage stage two-, four- and eight-cell embryos were equilibrated and frozen in 3.0 M EG supplemented with 0.25 M sucrose, the post-thaw viability was significantly lower ($p < 0.05$) than when more developed compact morulae or early-blastocyst embryos were frozen. Fifty-one per cent of two cell (43/84) and 47% of 4–8 cell (44/94) embryos developed further in culture. The highest survival and *in vitro* development rate was achieved for morula and early blastocyst embryos (56/70; 80%) ($p < 0.05$). Significantly, very low survival rate was obtained in the group of expanding and expanded blastocysts (10/59; 17%)

($p < 0.05$). These data indicate that the developmental stage in which mouse embryos are subjected to the rapid freezing protocol affects survival and development *in vitro*, and that the majority (80%) of morulae and early blastocyst stage embryos survive the procedure.

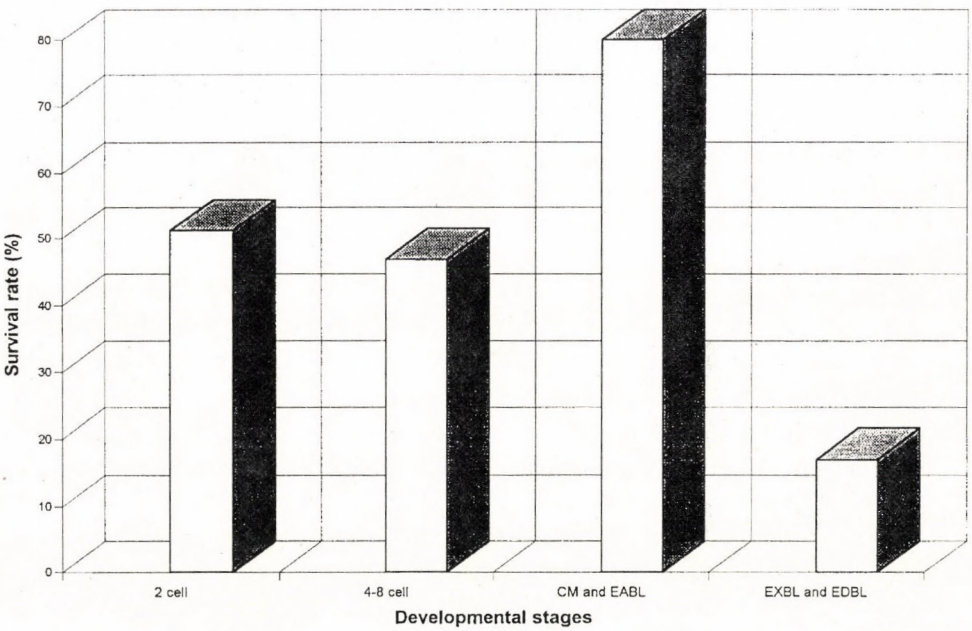


Fig. 3. Results of rapid freezing of mouse embryos at different developmental stages

Discussion

Mouse embryos at different developmental stages from two-cell to expanded blastocyst embryos were equilibrated with a high concentration of EG solution and frozen by direct plunging into LN_2 vapour for 2 min before immersing into LN_2, using a rapid freezing procedure. The quick freezing method is a combination of dehydration by non-permeating agents such as sucrose and simultaneous permeation of the embryo by the cryoprotectant such as EG which enables the cells to supercool to –170 or –196 °C without the formation of lethal intracellular ice (Leibo, 1989). In the cryopreservation of embryos using a rapid freezing procedure, a high concentration of cryoprotectant is required to give optimum protection. A prolonged equilibration period could have resulted in a very high intracellular concentration of EG making the embryos more sensitive to osmotic shock

caused by the rapid influx of water during rehydration after thawing (Leibo, 1989).

The results of this study indicate that the decrease in viability of embryos is not due to chemical toxicity or osmotic shock since the exposure of the embryos in 3.0 M EG with 0.25 M sucrose for 20 min without freezing did not detrimentally affect their capacity to develop *in vitro*.

In contrast, these data indicate that the developmental stage and the age of embryos are the determining factors for survival which also determines the efficiency of the freezing procedure. Using the same freezing technology, significant differences were found among the *in vitro* survival rates of embryos quick frozen at different developmental stages. In our hands, using 20 min equilibration, less two-cell (43%), four- and eight-cell (44%) stage embryos survived and developed *in vitro* than that obtained by Rayos et al. (1992*a*, 1992*b*) using 10 min equilibration.

No published data are currently available using the above described rapid freezing protocol for cryopreservation of later preimplantation stage embryos such as compact morulae and early, expanding and expanded blastocysts in a freezing medium containing 3.0 M EG and 0.25 M sucrose. We found that 80% of the compact morulae and early blastocysts developed in culture after rapid freezing in LN_2 vapour ($p < 0.05$). In contrast, we obtained a significantly lower survival rate (17%) in the group of expanding and expanded blastocysts ($p < 0.05$).

The results of this study indicate that EG in combination with sucrose can be used effectively as a cryoprotective agent in the rapid freezing of later preimplantation stage mouse embryos. The presented rapid freezing procedure can be strongly recommended for freezing of compact morulae and early blastocyst stage embryos because a high survival rate (80%) could be achieved. This procedure is simple and fast, only 22–23 minutes including the equilibration. The results of embryo survival are comparable to those obtained by conventional (Leibo, 1986; Niemann, 1991) and vitrification (Rall, 1987) methods.

Acknowledgement

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A COMPARISON OF RECTAL PALPATION AND ULTRASONOGRAPHY FOR THE EVALUATION OF SUPEROVULATORY RESPONSE IN BEEF HEIFERS

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The superovulatory response was followed up in 14 beef heifers by rectal palpation and by ultrasonographic examinations of the 28 ovaries, on day 7 after superovulation induced with PMSG (ovulation = day 0). Using a B-mode ultrasound instrument (Aloka Echo Camera SSD-210-DX II) equipped with a 5 MHz linear transrectal transducer, ultrasonography was found to be more reliable than rectal palpation. A significantly higher number of follicles ($P \leq 0.049$) was identified by ultrasound scanning than by rectal palpation of the ovaries: follicles were more easily differentiated from cysts using ultrasonography, since each fluid-filled vesicle was accurately measured on the screen and presented on the echogram.

Key words: Heifer, superovulation, superovulatory response, rectal palpation, ultrasonography

Transrectal ultrasonography is widely used in monitoring reproductive organs of cows and heifers. In addition to pregnancy diagnosis (Kähn, 1985; Kastelic et al., 1988; Herak et al., 1993) it is used for assessing physiological and pathological alterations of the reproductive organs, particularly ovaries (Kähn, 1986; Kähn and Leidl, 1986; Pierson and Ginther, 1988; Sirois and Fortune, 1988; Leidl, 1993; Herak et al., 1995).

More recently ultrasound scanning has been more often applied in monitoring superovulatory responses in embryo transfer (ET) programmes, along with gynaecological (vaginal and rectal) and endocrinological examinations. However, the application of diagnostic ultrasonography in the ET industry has not been explored extensively (Pierson et al., 1988).

Superovulation is still the most critical factor influencing the embryo yield. The efficacy of superovulatory treatment is estimated by the number of corpora lutea (CL), the rate of collected embryos and the number of transferable embryos.

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Following the development of methods for the non-surgical collection of bovine embryos, the number of corpora lutea and follicles after superovulation has been estimated predominantly by rectal examination. The number of corpora lutea is generally used for estimating the efficiency of the superovulatory treatment (Guay and Bedoya, 1981). The same authors report that assessment by rectal palpation prior to embryo flushing is subjective (and always underestimates the number of corpora lutea) and more difficult when superovulatory responses are excessive.

Pierson and Ginther (1988) suggested that ultrasound examination of the ovaries at the time of embryo recovery in order to determine the number of corpora lutea, thus the number of ovulated follicles, may result in maximal recovery rates. The flushing should be performed until the number of collected embryos approaches the number of CL determined ultrasonically.

Aria et al. (1993) claim that the determination of superovulatory responses in cattle exclusively by clinical examinations is subjective and unreliable, especially in those cases when the response is high. Consequently, they conclude that more objective data on the time of ovulation, the ovulation rate (the number of corpora lutea) and the predictable number of recovered embryos can be obtained by endocrinological examination and ultrasonography.

Leidl (1993) also suggests that the ovarian changes following superovulation, including the ovulation and subsequently the presence of luteal structures, may well be followed up by ultrasonography during the ET programme, which is practically impossible by rectal estimation.

Robertson et al. (1993) discussed several problems diminishing the efficacy of ultrasonography in monitoring the superovulatory response.

Inspired by those considerations, we decided to compare the superovulatory response on the ovaries of beef heifers, obtained by rectal palpation and by ultrasonography. In that way we could evaluate the usefulness of ultrasonography in the assessment of superovulatory response (number of corpora lutea, follicles, cysts), which is very important for obtaining a satisfactory number of transferable embryos.

Materials and methods

Fourteen beef heifers of Simmental breed (age: 12–14 months; body weight: 380–450 kg) were selected from a feedlot and transported to the Veterinary Clinic where they were kept during the experiment and their cycles were supervised daily. After 10 days of adaptation, heifers received half a briquette of Anifertil® per os (AniMedica; a briquette of 118 g contains 24 mg chlormadinone acetate (CAP), 200,000 IU vitamin A, 100 mg vitamin E-acetate) daily for 20 days, in

order to synchronise their cycles. At the time of expected oestrus (48 h after the last administration of briquette), heifers were examined gynaecologically (vaginal and rectal examination) and by ultrasonography. Follicular development was recorded by daily measurement on the monitor screen using callipers. When a follicle reached a minimum diameter of 13 to 15 mm, ovulation was induced by intramuscular injection of 2 ml of Gonavet® (VEB Berlin-Chemie; 1 ml contains 50 mg D-Phe-LHRH and 1 mg methyl-4-hydroxybenzoate). Ovulation was determined by rectal palpation and by ultrasound scanning of the ovaries, and 10 days after ovulation the superovulatory procedure was performed using PMSG (Folligon®, Intervet; each ampoule contains 1000 IU. of lyophilised serum gonadotropins). After 48 h, 2 ml of Cloprosin®-G (Krka, Novo Mesto, Slovenia; 1 ml contains 400 μg of synthetic prostaglandin $F_{2\alpha}$ analogue in the form of cyclodextrin-clatrate) was applied intramuscularly. Forty-eight h after prostaglandin administration, gynaecological (vaginal and rectal) and ultrasonographic examinations were performed again to determine superovulation and oestrus (Fig. 1).

All 14 animals were examined on day 7 following oestrus, first by rectal palpation and then by ultrasound scanning, to evaluate the superovulatory response on the ovaries (number of corpora lutea, follicles and cysts), and echograms were recorded. All liquid-filled vesicles measuring 5 to 20 mm in diameter, which appeared on the screen as spherical black (nonechogenic) areas, were classified as follicles. Corpora lutea were identified as spherical roughly-grained grey (echogenic) structures, well-demarcated from surrounding ovarian tissue. Cystic structures appeared on the screen as spherical black (nonechogenic) areas more than 20 mm in diameter (Fig. 2).

Ultrasound scanning was carried out using a B-mode ultrasound instrument (Aloka Echo Camera SSD-210, DX II) equipped with a 5 MHz linear transrectal transducer. During examinations each ovary was scanned several times on more than one plane and echograms were recorded on thermo-paper using electronic printer (Mitsubishi Video Copy AP-8600) connected to the ultrasound device.

The significance of the differences established in the superovulatory responses, and the diagnostic results estimated by rectal palpation and by ultrasound scanning were compared using χ^2-test and *t*-test, described by Petz (1985).

Results

By rectal palpation of the 28 ovaries, 88 corpora lutea, 16 follicles and 20 cysts were recorded, whereas by scanning the ovaries 99 corpora lutea, 46 follicles and 17 cysts were identified. The results obtained by each method of evaluation are given in Table 1 and Fig. 3.

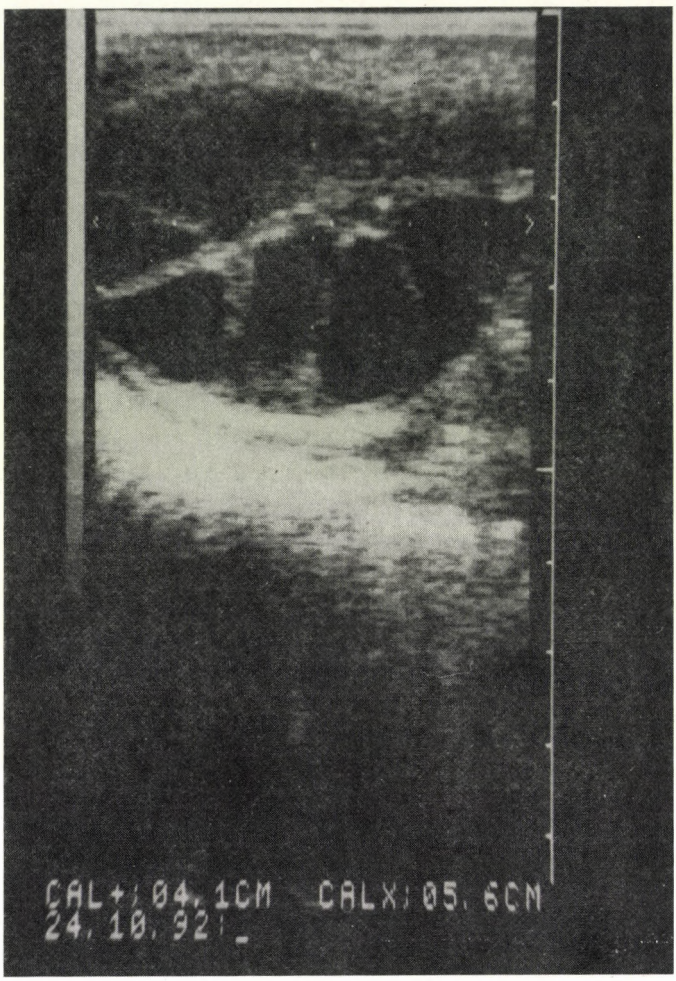


Fig. 1. Ultrasound image of the ovary superstimulated with PMSG. Boundaries of the ovary with follicles are marked by arrows

The data presented in Table 1 and Fig. 3 indicate that on day 7 of the superovulatory cycle, 16 ($\bar{x}$ = 1.14) follicles were diagnosed by rectal palpation whereas 46 ($\bar{x}$ = 3.29) were diagnosed by ultrasonography. The number of follicles identified by ultrasonography exceeded that diagnosed by rectal palpation by 30 (i.e. by 187.5%).

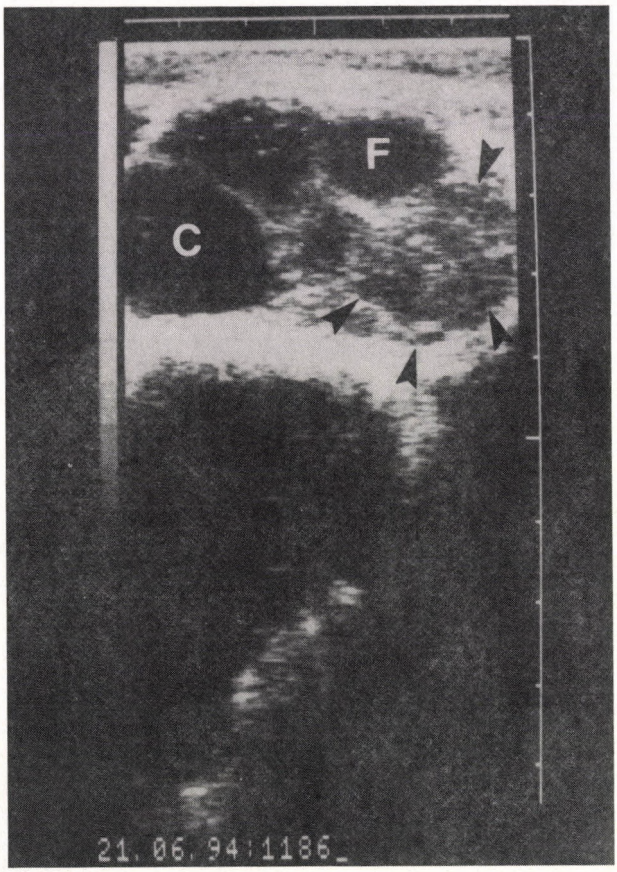


Fig. 2. Ultrasound image of the superovulatory response on the ovary on day 7 after oestrus. F = follicle; C = cyst. Corpus luteum is outlined by arrows

Using the χ^2-test, a significantly higher number of follicles was diagnosed by ultrasonography in relation to rectal palpation ($P \leq 0.049$). Similar results were obtained when the differences were analysed by the *t*-test ($P \leq 0.004$).

By rectal palpation 88 ($\bar{x} = 6.28$) corpora lutea were diagnosed; however, ultrasonography identified 99 CL ($\bar{x} = 7.07$), which is 11 (12.5%) more. No significant difference was established between rectal and ultrasonic assessment of the corpora lutea.

By rectal palpation 20 ($\bar{x} = 1.42$) cysts were diagnosed, whereas by ultrasonography 17 ($\bar{x} = 1.21$) cysts were identified. Three cysts (i.e. 15% of the cysts) diagnosed by rectal palpation could not be identified by ultrasound examination. No significant difference was established between these values.

Table 1

Differences in the efficiency of superovulatory response diagnosis on 28 ovaries from 14 heifers

Structures on the 28 ovaries	Follicles			Corpora lutea (CL)			Cysts		
	n	$\bar{x}$	SE	n	$\bar{x}$	SE	n	$\bar{x}$	SE
Rectal palpation	16	1.14	0.36	88	6.28	0.87	20	1.42	0.35
Ultrasound scanning	46	3.29	0.56	99	7.07	0.66	17	1.21	0.42
Animals (n)					14				

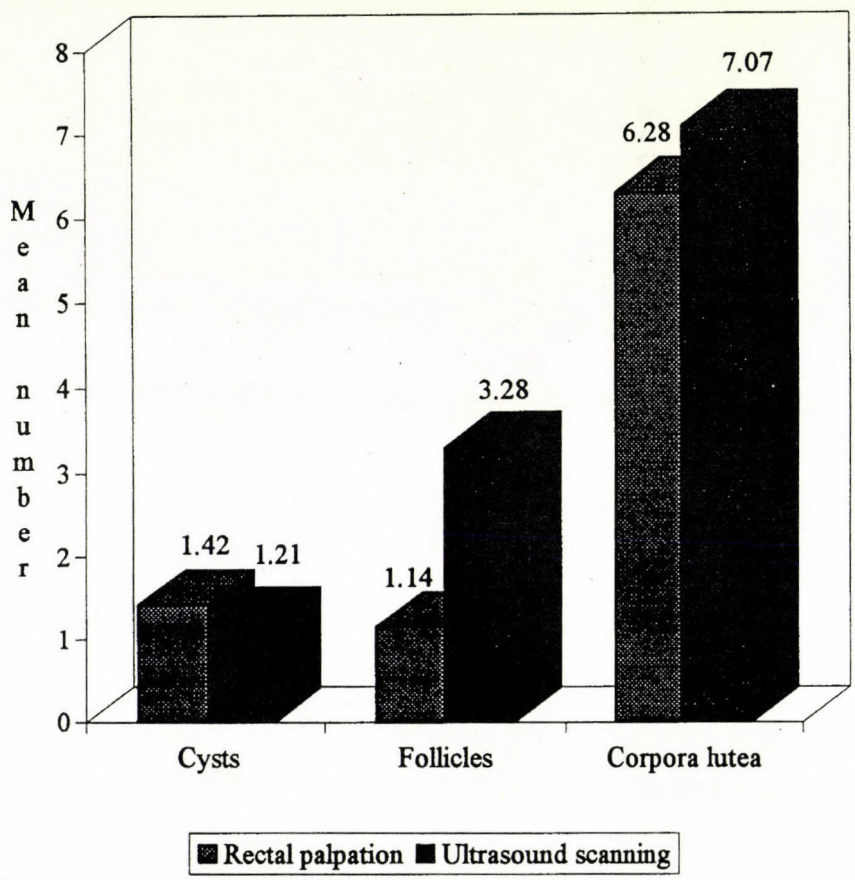


Fig. 3. Relation of the mean values of cysts, follicles and corpora lutea in the 14 superovulated heifers, i.e. 28 ovaries, attained by rectal palpation and by ultrasound scanning

Discussion

By the χ^2 test it was established that significantly ($P \leq 0.049$) higher number of follicles was recorded by ultrasonography compared to rectal palpation, whereas the number of cysts and corpora lutea on the ovaries, estimated by rectal and ultrasound examinations, did not show significant differences. The same results were obtained when the differences were analysed by *t*-test ($P \leq 0.004$). This can be explained by the fact that follicles <10 mm were not detectable by rectal palpation, which is consistent with the observations of Dawson (1975) and Guay and Bedoya (1981).

By ultrasound scanning we could identify follicles ≥5 mm in diameter, which appeared on the screen, like other liquid-filled vesicles, as spherical black (nonechogenic) areas (Fig. 1). Our findings are in accordance with the results obtained by Pierson and Ginther (1988), whereas Kähn and Leidl (1986) reported that follicles >10 mm in diameter could easily be detected and those measuring 3 to 5 mm could be identified with an error, when they lay close to one another. Kähn (1985) also established that follicles measuring 6 to 10 mm are diagnosed with insignificant error. Botz (1991) confirmed that in the course of superovulatory responses 31% more follicles were detected by ultrasonography than by rectal examination.

Although corpora lutea are easily palpable by rectal examination (compared to follicles), 11 (12.5%) more were identified by ultrasonography. In comparison, Botz (1991) reported that only 60% of the corpora lutea found by ultrasonography could be diagnosed by rectal palpation. Corpora lutea appeared on the screen as spherical, roughly-grained, grey (echogenic) structures, well-differentiated from surrounding ovarian tissue (Fig. 4). Similar findings were obtained by Pierson and Ginther (1988) who reported that corpora lutea formed after superovulation were individually discernible after day 3 or 4 by ultrasound scanning. Purwantara et al. (1994) also encountered a lower number of corpora lutea by rectal palpation than by ultrasound scanning. Robertson et al. (1993) stated that luteinised follicles cannot be distinguished from corpora lutea by ultrasonography.

It was more difficult to diagnose corpora lutea by rectal palpation when they were adjacent to one another and when several follicles or cysts were present on the ovaries, as reported by Guay and Bedoya (1981).

The 15% difference in the number of cysts detected by rectal examination and by ultrasonography was not significant, because fluid-filled structures of more than 20 mm in diameter were mainly detectable by rectal examination. Those cystic structures appeared on the screen as spherical black (nonechogenic) areas because they are fluid filled; we could measure them by ultrasonography and, therefore, non-significant differences in diagnosis were present only in cases when they

were on the border of 20 mm in diameter (Fig. 2). Dawson (1975) also considered all fluid-filled structures of more than 20 mm in diameter as cysts on the ovaries. By rectal palpation of the cysts in living animals and by inspection of those ovaries after slaughter, he found that the error of rectal assessment was 6.6%. Stolla and Himmer (1979) reported that it is difficult to determine differences between follicles and cysts by rectal palpation of the ovaries. Therefore, they concluded that assessment of the ovaries by rectal palpation is subjective regarding the size and consistence of fluid-filled structures. Küst and Schaetz (1983) reported that the basic principle to be observed in the practice is that fluid-filled structures exceeding 20 mm in diameter are no longer follicles but cysts.

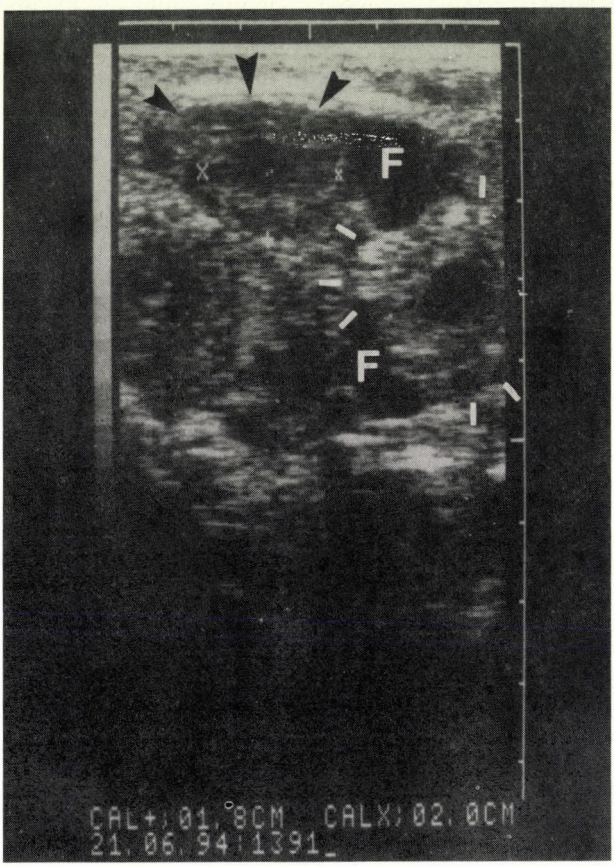


Fig. 4. Ultrasound image of the superovulatory response on the ovary on day 7 after oestrus. Corpus luteum is marked by arrows (size: 2.0 × 1.8 cm). F = follicles

In distinction from the rectal assessment of ovarian cysts, Kähn and Leidl (1986) and Pierson and Ginther (1988) reported that by ultrasonographic examination of the ovaries ovarian cysts can be simply and easily identified, and that fact is in accordance with our findings.

The main conclusions of this study are as follow: (1) A significantly ($P < 0.049$) higher number of follicles was identified by ultrasonography than by rectal palpation. (2) Follicles could be differentiated from cysts more easily by ultrasonography than by manual rectal assessment, since the former method enabled the accurate measurement of each fluid-filled structure.

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BOOK REVIEWS

H. Heinzel, R. Fitter and *J. Parslow:* **Pareys Vogelbuch. Alle Vögel Europas, Nordafrikas und des mittleren Ostens.** Seventh, completely revised edition. Parey Buchverlag, Berlin, 1996. 384 pages with over 3000 coloured illustrations and 600 maps, showing distribution of every bird regularly found in Europe, North Africa and the Middle East. Price: DM 38,- ISBN 3-8263-8121-1

An increasingly common challenge for veterinarians is the identification of different bird species in the practice. While few vets are occupied with zoo birds or with pets originating from the wild, there is an increasing demand for treatment of injured and captured wild birds, and veterinary inspectors also often have to check shipments of wild birds at the border. The education in zoology at veterinary schools is usually not comprehensive enough to provide knowledge about this topic in appropriate depth. Many veterinarians need, therefore, a quick reference that helps them identify their "patients". Such a helpful reference is the discussed field guide that was designed for people who wanted a comprehensive but quick identification guide to the birds of the Western Palaearctic. Both the text and the plates were designed for easy comprehension even by those who have no previous experience with birds.

Very shortly after the appearance of the new English edition, the popular field guide of H. Heinzel et al. is now available also in German translation. Due to language preference and closer links of Hungarian book distributors with German rather than British publishers, previous editions of this book were, for a long time, simply known as "the Pareys" amongst amateur and professional Hungarian ornithologists alike. Even though English is preferred to German as a second language amongst young people nowadays, this field guide will probably sell just as well in German as in English in Hungary. For those who do not have a problem with either of the two languages, the more expensive German edition might be a better choice since it is free of the minor but annoying spelling mistakes occurring in the English version.

The quality of bird identification guides largely depends on the quality and number of the pictures. The German-born artist, Herman Heinzel repainted all of the colour plates of the previous edition, thus eliminating the few existing errors, and also adding a number of new paintings. In average, about four different pictures show every species in different plumage with as much as sixteen for long-tailed duck, but even vagrants are depicted usually on two or three pictures. A new plate dealing with hybrid diving ducks is a special bonus compared to the previous editions. Almost every empty space is utilised by pictorial information but the artist and the designers of the book still found a balance by not making the plates overcrowded. A weak spot of the plates is, however,

the unnatural brightness of the blue on coracid birds (rollers and bee-eaters) and of the red on the breeding plumage of godwits. Even so, birds are easily recognisable even for beginners with the help of the plates.

The text of this edition was translated by Heinrich Hoerschelmann, taking over the position of the late Professor Günther Niethammer in the former edition. Not only did he pay special attention to correcting the misspellings occurring in various scientific names, but also diminished or eliminated a few existing inaccuracies in the text (see the "portlandica" type of common tern or the allegedly inseparable juvenile of lesser black-backed gull from herring gull in the English version). Even though it is very difficult to cram the identification features of different plumages and races of a species into 3 to 15 lines, the text is still a very useful accompaniment to the pictures.

Identification is often supported by the likelihood of the occurrence of the given species at the place of observation. The short description on habitat in the text, and acronyms referring to occurrence in Germany at the end of the description are also supported by colour distribution maps on breeding, migration and wintering range. The otherwise quite accurate maps are, in some cases (e.g. when showing the Canaries or the Azores as small spots with no reference to the mainland), hardly recognisable by the average reader without using the text due to their small size.

Nowadays there is keen competition on the market between different bird identification guides, and everyone can find a book that suits one's needs the best. Since veterinarians usually have limited time for identification, a book with visual information rather than one with lengthy descriptions is preferred. This need is probably fulfilled best by a German or English copy of this book.

Gábor Magyar

Manfred Heidenreich: **Greifvögel. Krankheiten, Haltung, Zucht.** Blackwell Wissenschafts-Verlag, Berlin–Wien, 1996. 294 pages with 334 illustrations and 31 tables. DM 298,- ISBN 3-8263-3090-0

Keeping or even breeding raptors has become very popular by these days. Even though possession and traffic of birds of prey is strictly regulated in most European countries by national and international laws and agreements, zoos, aviaries, falconers or repatriation centres of injured or confiscated birds more and more often seek the help of veterinarians. Since some of the bred and trained falcons have a market value of several thousand dollars, and the value of wild ones to be repatriated may simply be impossible to translate into any currency, the demand for the professional and effective treatment of their diseases does not need further explanation. On the other hand, while the knowledge on keeping, breeding and diseases markedly expanded in the past decades, a comprehensive work that veterinarians could reach for was, however, hard to find on the bookshelves until now.

The original intention of the authors was to write a book on diseases of falcons only. Since many of the diseases may be traced back to bad keeping practices or inap-

propriate diet, they soon realised that the topic of the book needed to be extended. Therefore, they attempted to include and cover as much as possible of the field of falcon breeding and keeping.

After a short introduction to systematics, a detailed chapter deals with the basics of keeping raptors. Anyone interested in this topic can find excellent coverage on the subject.

Feeding, breeding, identification and individual marking of falcons are discussed in the subsequent chapters. While feeding and breeding are of great importance to veterinarians and breeders alike, the authors probably thought that identification of different species was in the scope of taxonomists and bird-watchers rather than breeders, and even though the high quality and number of illustrations is maintained even here, the extremely complicated problem of identifying hybrids was kept to the basics in the text.

The clinical part follows the traditional arrangement of other veterinary books. The bacterial and viral diseases are discussed in the chapter of infectious diseases and followed by the description of parasitic ones. Alimentary diseases are also summarised in a separate chapter. Since raptors are on the top of the food chain, toxicology plays an extremely important role in their medicine. Thus, it well deserved a separate section in the book, as did systemic diseases and tumours. Well-designed tables give guidance on doses, side effects etc. during anaesthesiology of different raptor species.

Repatriation will be of special value to nature conservationists. Inclusion of this topic may be explained by the high nature conservation value of certain raptor species. It is just to be praised that all legal aspects of keeping falcons are also excerpted from the corresponding law sources and forensics is also treated in appropriate depth. Even if it focuses on German law, conventions on international trade (CITES) and EU directives are also important even for non-German readers. The between-the-chapters references are made complete by a bibliography at the end of the book, listing relevant works for further reading. Better communication between falconers and vets is enhanced by a glossary of the commonly used technical terms in German.

The content and outline of the book are in harmony and are both very attractive. The book found a good balance by providing comprehensive information for veterinarians, breeders and nature conservationists alike while keeping it as simple as possible so that it remains understandable for all readers with any kind of background education. It is a must for those veterinarians who treat raptors but it is strongly recommended for anyone dealing with falcons or for those who are novices in this field but would like to learn more about it.

Gábor Magyar

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ANNOUNCEMENT

IAMS Company Award 1996 at the WSAVA Congress in Jerusalem

Veterinary science is a rapidly developing field of knowledge and the veterinary practitioners have to rely on high standard publications in veterinary journals to keep up with the new developments in their profession. The IAMS Company is always at the forefront of new achievements in companion animal science and considers it her obligation to make this science available to the veterinary community. The Company wants to express its support for outstanding veterinary publications by awarding the best clinical articles that were published.

At the opening session of the WSAVA Congress in Jerusalem, dr. Peter Bedford, President of the WSAVA, has presented the IAMS Company Award 1996 to dr. Karine Onclin from the Department of Reproduction of the Veterinary Faculty in Liège, Belgium. The winning article, entitled *"Practical use of a combination of a dopamine agonist and a synthetic prostaglandin analogue to terminate unwanted pregnancy in dogs"*, was published in the Journal of Small Animal Practice (1996) **37,** 211–216. Co-author is dr. J. P. Verstegen from the same department. The article was chosen as the winner by a three-member independent committee of expert clinicians, out of more than 30 articles submitted and published between 1 July 1995 and 1 July 1996. Dr. Onclin was the guest of IAMS Pet Food International Inc. during the WSAVA Congress, received the check for the ECU 2500 prize with the certificate and made an excellent presentation of her study at the Congress.

For the IAMS Company Award 1997 papers published between 1 July 1996 and 1 July 1997 can be submitted before 1 August 1997 to the project coordinator Mrs. M. van der Kraan, IAMS Pet Food International Inc., Luchthavenweg 67, 5657 EA Eindhoven NL. One original reprint and three copies are requested plus the curriculum vitae of the first author.

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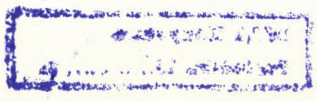
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