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Tomus XXVIII

INDEX

Bacteriologia

PESTI, L. and SEMJÉN, G.: *Escherichia coli* Vaccine for Prevention of Neonatal Enteric Colibacillosis in Pigs 1

PESTI, L. and SEMJÉN, G.: *E. coli* Heat-Labile (LT) Antitoxin Levels in Pigs and in *E. coli*-Vaccinated Sows 12

Virologia

Шврчек Ш., Александер, Р., Вртяк, О. Я., Мачичка, О. Грудих, И. и Ондрейка, Р.: Изоляция вируса бешенства из хомяка обыкновенного (Cricetus cricetus) в Чехословакии 21

Шврчек, Ш., Вртяк, О. Я., Герлахов, Д., Голы, Л., Анталик, Д., Александер, Р. и Ондрейка, Р.: Пероральная инфекция вирусом бешенства лисицы и кошки домашней 27

Шврчек, Ш., Втряк, О. Я., Селимов, М. А., Аксенова, Т. А., Герлахов, Д., Голы, Л., Дюран, А. и. Байова, В.: Экспериментальные предпосылки и техника пероральной антирабической иммунизации диких плотоядных. I. Предварительные опыты на лабораторных животных 33

Шврчек, Ш. и Вртяк, О. Я.: Модификация количественного метода ИПВЭ для определения иммуногенной активности антирабических вакцин в опыте на мышах ... 43

BELÁK, S.: Peroperties of Bovine Adenoviruses 47

Parasitologia

JUHÁSZ, S., GÁLFI, P. and MOLNÁR, K.: Data on the Proteolytic Enzyme System of *Lernaea cyprinacea* 57

Physiologia

SZEGEDI, B., JUHÁSZ, B., SZELÉNYI-GALÁNTAI, Marianna, JÉCSAI, Jolán and TELEKI, Mária: Experimental Studies on Early-Weaned Lambs. I. Development of Gastric Digestion and Metabolic Changes during Weaning 71

SZELÉNYI-GALÁNTAI, Marianna, JÉCSAI, Jolán, JUHÁSZ, B., SZEGEDI, B. and TELEKI, Mária: Experimental Studies on Early-Weaned lambs. II. Nitrogen and Amino Acid Metabolism 85

MURAI, T., PETHES, GY. and PÉCZELY, P.: Changes of Plasma Progesterone and Testosterone Level in Chicken *(Gallus domesticus)* from the First Day of Life to the Beginning of Oviposition 97

PÉCZELY, P., PETHES, GY., SZELÉNYI, Z. and MURAY, T.: Variations in Plasma Level of Sexual Steroids during the Oviposition Cycle in Laying Hens (*Gallus domesticus*) 103

HORVÁTH, Z. and SZEKERES, T.: Abnormal Elctrocardiograms in Leucotic Cattle 109

Bibliographia

Bacteriologia

CZEGLÉDI-JANKÓ, G. and DANKÓ, GY.: A New Microbiological Assay System for Antibacterial Druf Residues in Animal Organs. Estimation of Nigericin Residues 121

Virologia

MOCSÁRI, E.: Vaccination Experiments Against Transmissible Gastroenteritis (TGE) of Swine. VII. Immunoglobulin Characteristics of Antibodies in Milk of Sows Vaccinated with the „CKp" Strain of TGE Virus 131
KAPP, P. and LAPIS, K.: Reaction of Dogs Treated with Immunosuppressive Agents to Infection with the Virus of Rubarth Disease 147

Mycologia

PALYUSIK, M., HAGLER, W. M., HORVÁTH, L. and MIROCHA, C. J.: Biotransformation of Zearalenone to Zearalenol by *Candida tropicalis* 161

Physiologia

JÉCSAI, Jolán, SZELÉNYI-GALÁNTAI, Marianna, JUHÁSZ, B., SZEGEDI, B. and TELEKI, Mária: Experimental Studies on Early Weaned Lambs. III. Haematological Appraisal of Protein and Amino Acid Nutrition 169
TELEKI, Mária, JUHÁSZ, B., SZELÉNYI-GALÁNTAI, Marianna, JÉCSAI, Jolán and SZEGEDI, B.: Experimental Studies on Early-Weaned Lambs. IV. Energy and Gas Metabolism in Early-Weaned Lambs 179
HARASZTI, J., SZENCI, O., BÁRDOS, Anna and BAKSAI-HORVÁTH, Éva: Studies of Dietary Influence on the Health State of Preparturient Cows in Two Dairy Herds 191
HARASZTI, J., SZENCI, O., BAKSAI-HORVÁTH, Éva and SOLTI, L.: Peripartial Blood Profile Studies in High-Production Dairy Cows with Special Regard to Reproductive Re-activation 199
HAGLER, W. M., DANKÓ, Gy., HORVÁTH, L., PALYUSIK, M. and MIROCHA, C. J.: Transmission of Zearalenone and its Metabolite into Ruminant Milk 211
PALYUSIK, M., HARRACH, B., MIROCHA, C. J. and PATHRE, S. V.: Transmission of Zearalence and Zearalenol into Porcine Milk 219
VÁRNAGY, L. and IMRE, Rózsa: Teratological Examination of the Experimental Fungicide DPX 3217 50 WP in CFY Rats 225

Morphologia

VÁRNAGY, L.: Use of Recent Fetal Bone Staining Techniques in the Evaluation of Pesticide Teratogenicity 235

Bacteriologia

GERESSU, B. and TAKÁCS, J.: Identification of Genera in the Family of Enterobacteriaceae by a U-tube Multitest Method in Food Microbiology 241

Virologia

BODON, L. and PROHÁSZKA, L.: Isolation of an Adenovirus from Rabbits with Diarrhoea 247
MOCSÁRI, E. and HORVÁTH, Irén: Properties of the „CKp" Strain of Transmissible Gastroenteritis Virus. II. Studies on the *in vivo* Viral Replication in Newborn Piglets 257

Immunologia

SEMJÉN, G. and PESTI, L.: LT Antitoxin Titres in Three-Week-Old, *E. coli*-Vaccinated Pigs 269
PADÁNYI, M., RÉTHY, L., KULCSÁR, Á., GÉRESI, Magdolna, GELENCSÉR, F., POGÁNY, I., BÁCSKAI, L. and RÉTHY, L. A. Jr.: The Development of Primary Antibacterial Immune Protection against *Erysipelothrix rhusiopathiae* and the Effect of Anaerobic Corynebacterial Immunostimulants (Short communication) 273
RÉTHY, L., GÉRESY, Magdolna, GELENCSÉR, F., ÉCSY, Rita, PADÁNYI, M., POGÁNY, I. BÁCSKAI, L. and RÉTHY, L. A. Jr.: Heat-Inactivated Brucella Suspensions as Stimulants of Hosts' Defence against Tumour (Short communication) 277
GÉRESI, Magdolna, RÉTHY, L., ÉCSY, Rita, GELENCSÉR, F., PADÁNYI, M., POGÁNY, I., BÁCSKAI, L. and RÉTHY, L. A. Jr.: Antitumour Effect of Different Corynebacterial Immunostimulants in Non Isogenic Mouse-Tumour-System (Short communication) 281
GELENCSÉR, F., RÉTHY, L., BÁCSKAI, L., GÉRESI, Magdolna, POGÁNY, I., PADÁNYI, M. and RÉTHY, L. A. Jr.: The Effect of Anaerobic Corynebacterial Immunostimulants on the

Primary Immune Response to *Tetanus* Toxoid and *Perfringens* Epsilon Toxoid (Short communication) 285
SÓLYOM, F., FAZEKAS, Á. and CZELLENG, F.: Storage-Related Potency Changes in Monovalent Type O_1, A_5 and C Foot-and-Mouth Diseases Vaccine Preparations 289
TRAN DIHN TU: Effects of Antibiotics on the Immune System of Animals. I. Mouse Experiments with teh Low-Virulence VR_2 Strain of *Erysipelopthix rhusiopathiae* 297
TRAN DIHN TU: Effects of Antibiotics on the Immune System of Animals. II. Mouse Experiments with an Inactivated Adsorbed Vaccine against Swine Erysipelas 309
TRAN DIHN TU: Effects of Antibiotics on the Immune System of Animals. III. Experiments in Pigs Immunized with Live and Inactivated Swine Erysipelas Vaccines 317

Physiologia

KELLNER, Ágnes, SÁNDOR, I. und TAKÁCS, J.: Die Häufigkeit der exudativen (PSE) Fleischbeschaffenheit bei einigen heimischen Schweinerassen 333

Virologia

MOCSÁRI, E.: Physiochemical Properties of Field and Cell Culture-Attenuated Strains of Swine Transmissible Gastroenteritis (TGE) Coronavirus 341
FEHÉRVÁRI, T., GLÁVITS, R. and RÁTZ, F.: Studies on the Egg-drop Syndrome 351

Mycologia

SÁNDOR, Gabriella, VÁNYI, A. and PETRI, Ágnes: Effect of Irradiation on the Viability and Toxin Production of Different Fungus Species 361

Parasitologia

MOLNÁR, K.: Cutaneous Sphaerosporosis of the Common Carp Fry 371
ŠLAIS, J.: Experimental Infection of Sheep and Pigs with *Echinococcus granulosus* (Batsch, 1786), and the Origin of Pouching in Hydatid Cysts 375

Immunologia

SÓLYOM, F., PERENLEI, L. and ROITH, J.: A Live Attenuated Virus Vaccine Against Sheep Pox 389
BODON, L., ZSÁK, L. and NAGY, Éva: Detection of Netralizing Antibodies to Genuine Rabbit Adenovirus in Rabbit Antisera to Human Adenoviruses 399

Physiologia

MOUSTAFA, A. R. and MÉSZÁROS, I.: Interrelationship between the Total Protein Content of Bovine Seminal Plasma and Behaviour or the Spermatozoa after Freezing-and-Thawing 403
BOLDIZSÁR, H. and SIMON, F.: Effect of Zinc Sulphate Supplementation on Amino Acid Absorption from Pig Intestine. I. Intestinal Loop Tests 409
HARASZTI, J., SZENDI, O., SOLTI, L., FODOR, Éva and TÖRÖS, I.: Studies of the So-Called „Partus Syndrome" of Dairy Cows 419
RAFAI, P. and FODOR, Éva: Studies on Porcine Adrenocortical Function. I. Circadian Variations in, and Effect of ACTH Treatment on, the Paripheral Plasma Cortisol Level ... 433
RAFAI, P. and FODOR, Éva: Studies on Porcine Adrenocortical Function. II. Effects of the Ambient Temperature and Restricted Feeding on the Peripheral Cortisol Level ... 443

Morphologica

SZILÁGYI, M., B. KOVÁCS, A. and PÁLFALVI, I.: Relationship between the Ash Content and Microscopic Hardness of Swine Bones 455
KAPP, P. and SIMON, F.: Ultrastructural Cutaneous Changes in Zinc-Depleted Pigs 463

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ESCHERICHIA COLI VACCINE FOR PREVENTION OF NEONATAL ENTERIC COLIBACILLOSIS IN PIGS

By

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(Received October 12, 1977)

As reported earlier (PESTI and SEMJÉN, 1973), most (91.4%) of the *E. coli* strains isolated in Hungary from pigs died of *E. coli* diarrhea showed a positive reaction in the intestinal loop test, *i.e.* caused fluid accumulation in the ligated segments. In accordance with observations in other countries, the majority of the isolates were found to produce both heat-stable (ST) and heat-labile (LT) enterotoxins. Such strains, generally referred to as enterotoxic or enteropathogenic, proliferate in the anterior small intestine, where, in close association with the mucopolysaccharide layer of the intestinal mucosa, they produce enterotoxins. Oral infection with enterotoxic strains, or oral administration of ST and/or LT products of these, causes diarrhea in suckling pigs.

E. coli infections account for severe economic losses especially in large pig production units and to a lesser extent in smallholder herds.

The studies reported in this paper were carried out to develop a vaccine capable of conferring specific colostral protection in piglets through immunization of the sows against *E. coli* diarrhea. The LT as well as the ST enterotoxin of different strains is antigenically identical, irrespective of the serotype of the strain. It was therefore postulated that the anti-enterotoxic immunity elicited by vaccine-associated enterotoxins could protect against all enterotoxic *E. coli* strains.

Many vaccines, differing from one another in composition and application, have been developed against *E. coli* infections of pigs.

WILSON and SVENDSEN (1971) prepared a vaccine partially inactivated with formalin. KOHLER (1974) had fed live *E. coli* organisms to sows during the terminal days of pregnancy, and found that the offspring was highly resistant to challenge with the homologous live strain. RUTTER and JONES (1973) reported good results of vaccination with partially purified K88 antigen. VARGA (1976) used an $Al(OH)_3$-adsorbed and Freund's adjuvant-containing preparations. CARDELLA et al. (1974) obtained good results with certain not nearer described "bacterins". HILL and AKKERMANS (1976) evaluated vaccination trials conducted in the Netherlands. It may be concluded that the vaccines hitherto employed have been less immunogenic than expected.

DOBRESCU et al. (1974) were the authors who used an enterotoxin vaccine against *E. coli* diarrhea of neonatal pigs.

In our vaccine trials launched in a large pig production unit, we immunized a large group of sows and followed up *E. coli* diarrhea incidence among the offspring of vaccinees and non-vaccinees. The *E. coli* strains occasionally isolated from piglets diseased in, or succumbed

to, *E. coli* diarrhea, were typed. Hyperimmune sera were produced in pigs and were examined for neutralizing effect on the loop-dilating activity of live, enterotoxic *E. coli* strains and LT enterotoxins. The bacterium counts in the ligated intestinal segments were regularly determined. Piglets farrowed by vaccinated and non-vaccinated sows were challenged with enterotoxin preparations.

Materials and methods

Description of the vaccine. Standard 0141: K85ab, K88ab and 0149: K91, K88ac *E. coli* strains producing great amounts of both ST and LT enterotoxins were used as vaccine strains. Agar-agar was added as adjuvant and 0.5% formalin was used for inactivation. The vaccine contained, besides the inactivated enterotoxins, 500 million *E. coli* cells/ml.

Preparation of hyperimmune serum. Growing pigs 40—70 kg in bw were vaccinated with rising doses (range: 5 to 15 ml) of the vaccine, generally 9—12 times, at 4—5-day intervals, i.m. A blood sample was taken 2 weeks after the last immunization to assess then anti-enterotoxic titre. If this was satisfactorily high, the donor was exsanguinated.

The antiserum for the toxin neutralization tests was prepared in the same way, except that the immunizing vaccine contained the toxic product of only one strain, *viz.* the 0141: K85ab, K88ab.

Vaccination of the sows. Five ml vaccine was administered i.m. twice, *viz.*, 30 and 15 days before parturition.

Preparation of enterotoxins. LT and ST enterotoxins were obtained as described earlier (PESTI and SEMJÉN, 1973), following the methods of SMITH and GYLES (1970).

Inactivation tests with formaldehyde. To ST and LT enterotoxins prepared from the vaccine strains, formalin was added up to 0.5% final concentration. The toxins were examined for loop-dilating effect in isolated pig and rabbit intestinal segments after 24-hr exposure and subsequent 48-hr dialysis.

Intestinal loop tests in pig and rabbit. The test was described in detail elsewhere (PESTI and SEMJÉN, 1973). Seven to 12 weeks old pigs were used and, on the average, 15 intestinal segments were isolated in each. The loop-dilating effect was calculated as w/w ratio, *i.e.* weight of fluid-accumulating segment minus weight of empty segment per weight of empty segment.
All indices were calculated by this equation. According to comparative measurements, the index value thus obtained roughly corresponds to the quotient of fluid accumulation in ml and segment length in cm (ml/cm).

The rabbit tests were performed with the so-called "multiloop" procedure proposed by PIERCE and WALLACE (1972). Rabbits of 1.7—2.5 kg bw, fasted for 48 hr were used; 17—21 isolated segments were prepared per animal. One ml of the LT enterotoxin preparation was injected into each segment. Twenty-four hours later the animals were killed and the loop reaction was evaluated as in pigs.

Ligated loop bacterium flora studies. The counts of *E. coli* and other major components were determined in selected isolated segments as described elsewhere (PESTI, 1963). One ml content was taken, carefully to avoid contamination, from each dilated segment and 0.1 g mucus mixed with chyme was collected from selected empty segments as sample.

In neutralization tests varying doses (2—6 ml) of antisera were mixed with 0.5, 1 and 2 ml of LT preparations, 2, 4 and 8 ml of ST preparations or 1 ml volumes of 24-hr nutrient broth cultures of living organisms (dilutions 10^{-2} and 10^{-3}), 1 hr before they were injected into ligated segments. The mixtures were incubated at 37 °C. The dose variations are noted in the text. The control and serum-treated samples were injected into adjoining loops.

Challenge experiments. Twenty, 24- to 72-hour old, piglets farrowed by vaccinees and non-vaccinees were treated with 30 ml ST or LT preparations orally, through a gastric tube. Part of the animals were additionally given a peroral dose (30 ml) of hyperimmune serum. After treatment, the piglets were returned to their mother and the presence or absence of diarrhea was recorded.

Vaccination trial in large pig production units. The trials were commenced in January 1976 in the pig units of 3 state farms and one cooperative farm. The results obtained in one state farm trial are reported in this paper.

On a total, 361 sows were treated with the vaccine and 376 were left untreated to serve as control. Vaccinees and non-vaccinees farrowed a total of 6617 piglets. These were observed until 10 days of age, and the numbers of those diseased in, or succumbed to, *E. coli* diarrhea were recorded in both groups. The vaccination trial included two periods in winter and one period in summer. The experimental animals were kept in 4 completely isolated housing units, and the treated and control sows were either distributed randomly within the population of one house, or each in an isolated sow house stocked by animals of similar immunological status, age and farrowing term prognosis.

Results

Loop-dilating effect of live E. coli *cultures and enterotoxins.* E. coli *counts in the presence of hyperimmune serum*

Loop flora. The effects of the vaccine and of the corresponding hyperimmune serum were studied in ligated segments of pig and rabbit intestines.

Before these experiments the bacterium flora of selected ligated loops (so-called segmental flora) was examined. The results are shown in Table I. The pre-inoculation samples of segment contents were usually free of haemolytic *E. coli*, apart from very rare exceptions, whereas high *E. coli* counts were always found after inoculation with live, loop-dilating (enterotoxic) strains.

Table I

Bacterium counts in treated and untreated intestinal segments 1×10^6 germs/g segmental content = 1.00

	13 segment-positive strain, live culture n = 27	7 segment-negative strain, live culture n = 19	0141 ST enterotoxin n = 15	LT enterotoxin of several strains n = 13	Segments not treated n = 33
*E. coli**	420 (20—1000)	2000 (36—9300)	340 (24—1400)	740 (50—1600)	590 (11—1100)
	26	19	14	13	26
Enterococci	26 (0.40—100)	0.29 (0.03—0.80)	4 (8—120)	140 (0.80—520)	35 (0.04—170)
	13	3	7	13	15
Aerobic lactobacilli	150 (0.32—800)	0.62 (0.04—1.20)	22 (2.40—140)	28 (0.16—160)	35 (0.28—300)
	11	2	7	12	12
Clostridia	14 (2—26)	0	2 (0.20—4.0)	3.1 (0.01—8.0)	0.6 (0.002—5.0)
	2	0	4	8	9
Bacteroides	0	1.6 (0.80—2.40)	1.8 (1.20—2.40)	2.00	0
	0	2	2	1	0

*Figures in the top row show the mean and limit values of germ count, those in the bottom row the number of positive segments.

The isolated strains were haemolytic and serologically identical with the inoculum. High *E. coli* counts were also found in the segments inoculated with strains causing no fluid accumulation. Among the strains isolated from such (negative) segments some were haemolytic, others not. The *E. coli* counts were high in the enterotoxin-treated and in the non-inoculated control segments, but the isolates obtained from them were, for the most part, non-haemolytic.

Table II

Loop dilating effect and germ counts of live *E. coli* cultures in isolated pig intestinal segments, with or without type 0141 hyperimmune sera present

Broth culture (24 h, 1 ml)		Hyper-immune serum	Index	*E. coli* counts in isolated intestinal segments $\times 10^8$/ml
Serogroup	Dilution			
	10^{-2}	—	7.65	3.74
0141	10^{-2}	+	2.25	1.02
	10^{-3}	—	1.52	43.2
	10^{-3}	+	0.46	3.38
	10^{-2}	—	6.25	13.5
0149	10^{-2}	+	2.60	5.34
	10^{-3}	—	3.43	42.2
	10^{-3}	+	0.86	21.3
	10^{-2}	—	4.20	3.22
0147	10^{-2}	+	1.38	1.74
	10^{-3}	—	1.84	42.0
	10^{-3}	+	0.35	10.4

The lack of fluid accumulation in the untreated segments indicates that the positive secretion in the segments inoculated with enterotoxic *E. coli* strains is due to the enterotoxin.

Fluctuations in the counts of enterococci, lactobacilli and clostridia did not follow any definitive rule. The lactobacillus counts occasionally rose markedly in certain segments, while they were zero in others. Lack or multiplication of lactobacilli probably depended on their presence or absence in the given segment before ligation. Staphylococci, aerobic spore-bearing bacteria, fungi and bacteroides were variably present at low counts.

Neutralization of the loop-dilating effect of live E. coli *cultures.* The hyperimmune serum prepared with the vaccine was added to broth cultures of sero-

logically homologous and heterologous *E. coli* strains. The results are shown in Table II. All control segments accumulated fluid on inoculation with serum-free cultures up to 10^{-2}—10^{-3} dilution, while little or no fluid accumulated in segments injected with serum. The antiserum showed equal efficiency in preventing or depressing the loop-dilating effect of homologous and heterologous strains.

Table III

Neutralization of LT with *E. coli* 0141 hyperimmune serum in pig intestinal loop

Materials administered into ligated segment				Neutralization	Index
LT	ml	serum	ml		
0141 K85ab K88ab	2	—	—		15.92
0141	2	0141	2	+	0.33
0141	1	—	—	+	9.50
0141	1	0141	2		0.23
0141	0.5	—	—	+	6.50
0141	0.5	0141	2		0.15
0147 K89 K88ac	2	—	—		16.00
0147	2	0141	2	+	0.55
0147	1	—	—		10.10
0147	1	0141	2	+	0.33
0141	2	—	—		7.16
0141	2	negative serum	2	—	2.40

The *E. coli* counts did not differ markedly between segments inoculated with broth culture alone and broth culture + serum. Inter-segmental variation was within one logarithmic order in both control and serum-treated series. The data shown in Table II permit the conclusion that the antiserum neutralizes the loop-dilating effect of live strains without reducing the intra-segmental germ count.

Neutralization of the LT enterotoxin. The LT-neutralizing effect of the hyperimmune serum was measured at different toxin volumes. The results of a representative experiment are shown in Table III. The neutralizing effect of the serum was complete, irrespective of the homologous or heterologous origin of the LT-preparation and the response was dose-dependent. The negative serum preparation had a low protective effect.

Neutralization of the ST enterotoxin. The neutralizing effect of the hyperimmune serum on ST was tested in 14 pigs. Neutralization was observed in 8 pigs; (data not shown), in the remaining 6 the hyperimmune serum depressed fluid accumulation scarcely, if at all.

Vaccine trials

The effect of formalin. Data in Table IV clearly indicate that both enterotoxins became inactivated by the formalin treatment. Formalin itself acted as loop dilator. Since formalin is dialysable, absence of loop-dilating effect with the formol-treated and subsequently dialysed enterotoxins has presented

Table IV

Effect of 0.5 % formalin on *E. coli* enterotoxins

Material administered into ligated intestinal loop	ml	n	Index
0141 ST, dialysed	15	3	8.38
0141 ST, 0.5% formol-treated, dialysed	15	3	1.68
0141 LT, dialysed	1	6	11.16
0141 LT, 0.5% formol-treated, dialysed	1	4	0.17
ST medium control	15	2	0.42
ST medium control + 0.5% formol	15	3	6.75
Saline	15	4	0.12
Saline + 0.5% formol	15	2	6.06

Table V

Challenge with ST and LT in 1—3 days old offspring of vaccinated and non-vaccinated sows

	ST		LT		ST + serum		LT + serum	
	+	—	+	—	+	—	+	—
Non-vaccinee offspring	3*	0	3	0	0	3	2	1
Vaccinee offspring	1	3	0	4				

* number of pigs with diarrhoea.

Table VI

Protection of newborn pigs against *E. coli*-

Group No.	Vaccinee offspring group						
	No. of sows	No. of piglets farrowed	Piglet losses until 10 days of age				No. of litters with *E. coli*-diarrhea incidence
			Total	*E. coli*-diarrhea			
				No.	per cent incidence relative to		
					pigs born	pigs lost	
I	65	600	76	7	1.16	9.21	2
II	198	1631	148	6	0.36	4.05	7
III	98	959	47	4	0.41	8.51	2
Totals	361	3190	271	17	0.53	6.27	11

unequivocal evidence of formalin-induced transformation to toxoid of both LT and ST.

Challenge experiments. The results of challenge in two offspring groups farrowed by vaccinated and non-vaccinated sows, respectively, are shown in Table V. While all non-vaccinee offspring developed diarrhea on treatment with ST and LT enterotoxins, all vaccinee offspring, except a single ST-treated piglet, remained healthy. Oral protection by the hyperimmune serum was tested in the litter of an unvaccinated sow. All three piglets treated simultaneously with the serum and ST remained free from symptoms throughout the period of experiment. The scouring effect of LT was only partially blocked by the hyperimmune serum.

Field trials. The results of immunization experiments conducted in a large pig unit are shown in Table VI. Among 3190 vaccinee offspring 17, while among 3427 non-vaccinee offspring 105 died of *E. coli* diarrhea, covering 6.27 and 30.34% of total mortality, respectively. Litters with diarrhea incidence were 6 times more numerous in the non-vaccinee offspring group compared to vaccinee offspring, and mortality from the disease was lower in both offspring groups (4.05%, and 24.47%) during the summer season (group No. II). In the small intestines of both vaccinee and non-vaccinee offspring type 0147 and 0149 *E. coli* strains predominated.

It can be seen that, although the vaccination could not completely prevent the incidence of *E. coli* diarrhea, it did reduce litter involvement and case fatality by 4/5.

The strains isolated from the carcases belonged to serogroups 0149 and 0147.

diarrhea by vaccination of the sows

Non-vaccinee offspring group						
No. of sows	No. of piglets farrowed	Piglet losses until 10 days of age				No. of litters with *E. coli*-diarrhea incidence
		Total	*E. coli*-diarrhea			
			No.	per cent incidance relative to		
				pigs born	pigs lost	
60	519	78	35	6.74	44.87	27
214	1932	192	47	2.43	24.47	27
102	976	76	23	2.35	30.26	12
376	3427	346	105	3.06	30.34	66

Conclusions

The efficiency of the vaccine was tested in laboratory and field trials.

Preliminary experiments have shown that changes of the loop flora play no notable role in the fluid accumulation, for the counts of the different segmental flora components were unrelated to the degree of loop dilatation. Furthermore, assessment of segmental *E. coli* counts under different conditions has unequivocally indicated that production of enterotoxin rather than the increase in *E. coli* count is responsible for the loop-dilating effect (see Table II).

KOHLER (1967) and MINIATS et al. (1970) attributed the protective effect of their antisera to neutralization of the enterotoxins, while SMITH and LINGOOD (1971) as well as SMITH (1972) concluded from infection experiments for an antibacterial rather than antitoxic protective mechanism. This accorded well with SMITH and GYLES' (1970) finding that the loop-dilating effect of a live *E. coli* strain could be neutralized exclusively by a homologous antiserum. It should be noted that the last-cited authors prepared the antiserum by serial treatment with the same live strain, whence the serum was essentially an OK serum. Our hyperimmune serum, on the other hand, was raised by an *E. coli* vaccine in which enterotoxins were abundantly present. The absence of dilatation in the serum-treated segments without any appreciable reduction of the *E. coli* count compared with the control segments strongly supports the antitoxic nature of the serum-conferred protection. Further proof in support of an antitoxic mechanism emerged from our LT-neutralizing experiments and from the fact that the ST-effect was depressed or prevented by the serum, too.

Data in Tables II and III clearly indicate the intertypic identity of LT.

The experimental findings on the high efficiency of the vaccine have been confirmed by field experiences. Although vaccine treatment of the sows did not completely prevent *E. coli* diarrhea incidence among piglets, it accounted for a considerable reduction of losses and litter involvement. Morbidity and mortality rates were reduced to 1/5—1/6 of those for the comparable control. The available experience suggests that *E. coli* vaccination is advisable before each farrowing. Statement of its necessity requires further supporting evidence.

We have questioned the practicability of the oral immunization procedure proposed by several authors. This would presuppose daily treatment of the piglets with massive oral doses of *E. coli* from birth until 8—10 days of age (SCHIMMEL et al., 1975). The formolized vaccine described by WILSON and SVENDSEN (1971) also contains live organisms, which is a reason for objecting its field use. The same applies to KOHLER's (1974) otherwise good idea that the pregnant sows should be infected with live enterotoxic strains before farrowing.

Titrations of LT and other studies carried out during the vaccine field trial will soon be reported.

Our hyperimmune serum prevented the effect of ST in 8 of the 14 treated animals. DOBRESCU and HUYGELEN (1973) reported ST-protective effect of sera from LT-immunized pigs. The ST may well be a hapten-like substance capable of combining with other protein(s) to an antibody-stimulating unit in the vaccine preparation (PESTI and SEMJÉN, 1976). Our hyperimmune serum failed to fully protect non-vaccinee offspring piglets against challenge with LT, probably because the applied LT dose was too high (30 ml) relative to the serum dose. Full protection against LT challenge has been observed, however, in vaccinee offspring which may well have acquired a greater reserve of passive antibodies through regular intake of colostrum.

Summary

Treatment of pregnant sows with an adjuvanted formalin-inactivated *E. coli* vaccine containing LT, ST enterotoxin and bacterial cells was followed by reduction of the incidence of piglet *E. coli* diarrhea to a very low level relative to the morbidity for non-vaccinee offspring.

The passive protection conferred upon the piglets via colostrum is presumably antitoxic in nature and as such it is independent of the serotype of the producing *E. coli* strain(s), i.e. the vaccine is protective not only against homologous, but also against heterologous, strains of *E. coli*. Development of an adequate heterologous immune response was shown experimentally by intestinal loop test, using for neutralization a hyperimmune serum raised with the vaccine. The protective effect of this serum was unrelated to the *E. coli* count and other segmental flora components in the isolated loop.

The hyperimmune serum neutralized the loop-dilating effect of live *E. coli* as well as of the enterotoxin LT, and partially neutralized the ST enterotoxin. Formalin treatment transformed the LT and ST components of the vaccine to toxoid.

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E. COLI HEAT-LABILE (LT) ANTITOXIN LEVELS IN PIGS AND IN *E. COLI*-VACCINATED SOWS

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Treatment of sows with an *E. coli* vaccine containing enterotoxin was followed by considerable reduction of the incidence of *E. coli* diarrhea among the piglets. This suggested a major pathogenic role of enterotoxins in the disease and a primarily antitoxic nature of the immune response (PESTI and SEMJÉN, 1980).

Newborn pigs are protected against *E. coli* diarrhea passively *via* maternal colostrum. Serum and milk from vaccinated sows were shown to protect germfree piglets against the disease (SVENDSEN and WILSON, 1971). Anti-enterotoxin was demonstrated by intestinal loop test in the sera of both vaccinated and unvaccinated sows (WILSON et al., 1971), LT antitoxin was not titrated. Hyperimmune serum to *E. coli* was found to protect experimentally infected piglets by a presumably non-bacteriocidal mechanism (KOHLER and BOHL, 1966).

Antibodies to LT were found in the sera of 8—12 weeks old pigs (GYLES, 1974) and of unvaccinated pigs (WHIPP and DONTA, 1976).

The principal aim of the present studies was to assess, by a reliable titration procedure, the effect of our vaccine preparation on the LT antitoxin. Serum samples of unvaccinated sows and other swine were examined for the presence or absence of LT antitoxin and the anti-LT levels in vaccinees and non-vaccinees were compared.

Attempts were also made at detecting a correlation between age and LT-antitoxic level, and at confirming the antitoxic nature of the passive protection by immune absorption test.

Materials and methods

E. coli *enterotoxin*. LT enterotoxin was prepared from a type 0141: K85ab, K88ab strain as described previously (PESTI and SEMJÉN, 1973). The same batch of LT preparation was used throughout.

Sera. Paired serum samples were collected from 28 sows belonging to the production herd of a large pig unit in which a vaccination field trial had been carried out. The first blood sample was taken before vaccination, 30 days prior to the expected parturition; the second sample 1—3 days after farrowing. Samples were also collected from further 71 pigs of various ages, belonging to the herds of 4 state farms. Hyperimmune serum was prepared by serial inoculation with the *E. coli* vaccine (PESTI and SEMJÉN, 1980).

Milk samples. Pooled samples were collected from different teats of 10 vaccinated and 4 unvaccinated sows. Colostral whey was collected as proposed by SVENDSEN and BROWN (1973).

Intestinal loop test in rabbits. Toxin and antitoxin were titrated in ligated small-intestinal loops of New Zealand rabbits weighing 1.7—2.5 kg, after fasting for 48 hr. The so-called multi-loop technique, described by PIERCE and WALLACE (1972) was employed. In each animal 17—21 test segments, separated by 3 cm-long untreated portions, were prepared. Different toxin dilutions were injected into the loops in 1-ml volume, the toxin-serum mixtures in 2-ml volume. The results were read 24 hr after the intervention, and were expressed by an index (w/w quotient, accumulated segment fluid wt per segment wall wt), regarding values above 1 as positive.

Titration of toxin and antitoxin. Enterotoxin was titrated by the method of BURROWS and MUSTEIKIS (1966). The toxin was diluted in twofold serial steps, and 6—14 intestinal loops were inoculated for determination of the mean index value at each level of dilution. The index values were used to construct a dose-response curve, from which the ED_{50} value of the toxin was determined. The ED_{50} (1 toxic unit) so calculated corresponded to 50 μl.

The antitoxin levels of paired sera were determined by a slightly modified procedure of KASAI and BURROWS (1966). Serial twofold serum dilutions were prepared and 4 ED_{50} of enterotoxin was added in 1 ml volume to each dilution. After incubation for 1 hr at 37 °C, the serum-toxin mixture was inoculated into rabbit intestinal loops. An appropriate control was used with each test. All 28 serum pairs were so titrated, and each sample was subjected to preliminary approximative titration. Depending on the preliminary results, the neutralization test was omitted in some cases, while in others certain dilutions were titrated several times.

The arithmetical means of the incidence obtained in each dilution step of pre- and postvaccination sera were calculated and used to determine the neutralization coefficient (NC) by the formula

$$NC = 1 - \frac{X}{Y},$$

where X indicates the mean value of indices obtained at the given level of serum dilution, and Y is the upper asymptote read from the corresponding dose-response curve. The value of NC ranged between 0 (= no protection) and 1 (= full protection). Since all titrations were carried out with 4 units of toxin present, an NC value of 0.5 signified the neutralization of 3 units of toxin. With the NC values plotted out in a log-log system, two curves were constructed from which the antitoxin titre of the sera could be read directly at NC = 0.5. Thus, we modified KASAI and BURROWS's original antitoxin

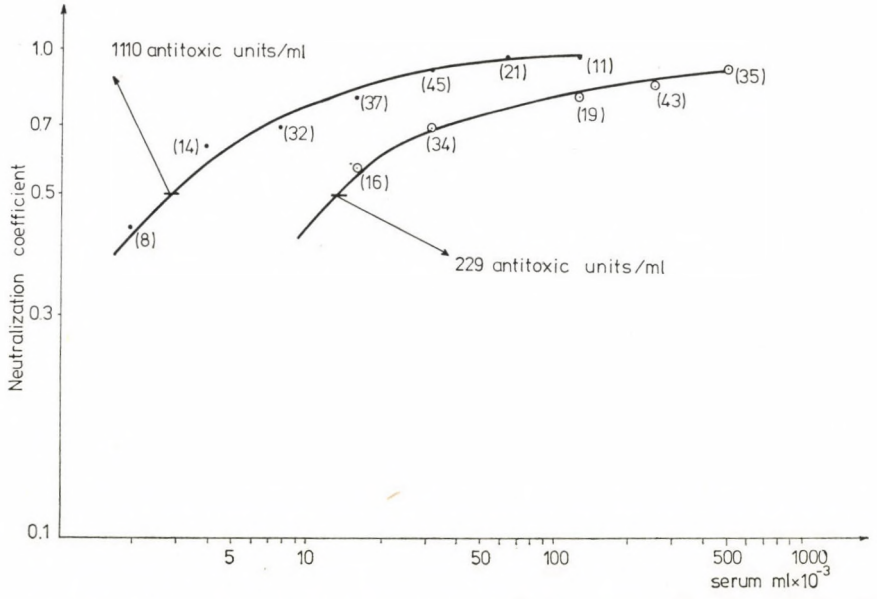
titration procedure only inasmuch as we considered the "whole" of pre- and post-vaccination sera instead of individual samples.

The 99 individual serum samples and 14 colostral whey samples collected from swine in different herds were titrated for economic reasons, by a simpler procedure. The reciprocal value of the highest serum dilution still producing full neutralization of the loop-dilating effect of exogenous toxin (4 ED_{50}) was multiplied by 4 (with regard to the 4 units of toxin added to the serum dilutions). The result corresponded to the number of antitoxic units present in 1 ml serum.

Results

The NC values obtained for the different dilutions of paired sow sera are shown in Fig. 1.

LT-neutralizing antibodies had already been present in the pre-vaccination samples, but their levels rose notably after vaccination. The NC of post-vaccination samples still approximated 1 (NC = 0.92), at as a high dilution as 1: 32, while the prevaccination samples had a similar protective value (NC = 0.91) only at 1: 2 dilution. The mean antitoxin contents of the paired sera were also calculated from the curves: it proved to be 229 and 1110 antitoxin units/ml for pre- and postvaccination samples, respectively. The data



Fig, 1. Titration of pre- and post-vaccinated sow sera for LT antibodies by rabbit intestinal loop test

shown in Fig. 1. clearly indicate that the vaccination caused a considerable, approximately fivefold, rise in the basic LT-neutralizing antibody levels.

One of the hyperimmune sera prepared in this laboratory with type 0141 and 0149 *E. coli* strains contained 3528 LT antitoxin units/ml as assessed by KASAI and BURROWS' procedure. This was roughly three times as high as the LT-antibody level in the sera of vaccinees. Accordingly, serial treat-

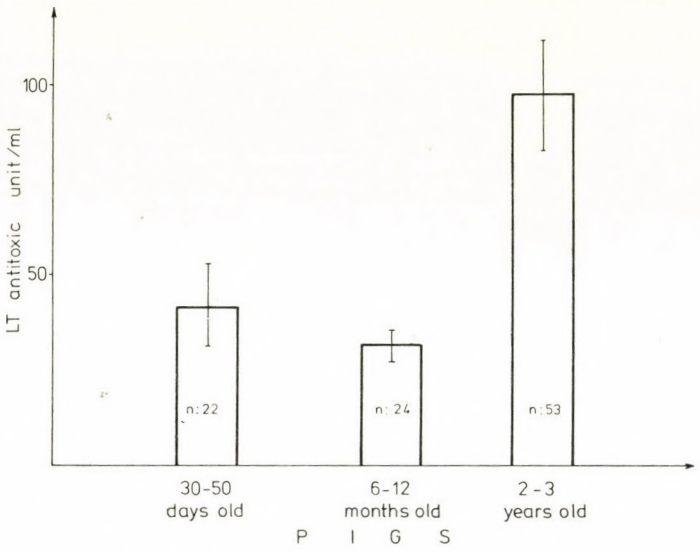


Fig. 2. LT-antitoxic titres in sera obtained from pigs of different ages in 4 farms

ment with our *E. coli* vaccine on 8 to 12 occasions at intervals of 4—5 days is the method of choice for the preparation of very strong LT-antitoxic sera. The hyperimmune sera so obtained proved to be suitable for oral protection of suckling pigs against oral infection with LT (PESTI and SEMJÉN, 1980).

The hyperimmune serum was used therapeutically, in 20 piglets 8—12 days of age, suffering from *E. coli* diarrhea. Of the 20 animals so treated 50% recovered. The serum was applied i.m., in 10 ml individual dose. Further studies are in progress about the therapeutic efficiency of the serum.

Fig. 2. shows the LT-antibody levels of the swine sera collected in 4 different farms. The serum samples taken from piglets 30—50 days of age contained 42.3 antitoxin units/ml and approximately the same value was found for 6—12-months old pigs. The inter-group difference was not significant statistically. By 2—3 years of age the LT-antibody level had reached 98.8 units/ml. These values, obtained by our modified procedure, are lower than those calculated by means of the neutralization coefficient. Nevertheless, the modified method reliably informs about the mean titre range, and requires

relatively few rabbits. Fig. 2 also shows that the LT-antibody level tended to increase with progressing age.

The colostral whey samples of vaccinees and non-vaccinees were tested for LT-antibody level by the simplified method. Fig. 3 shows that antibodies to LT were also present in the colostrum of unvaccinated sows (50 LT antitoxic units/ml). The colostral whey of vaccinees contained 288 LT-antitoxin

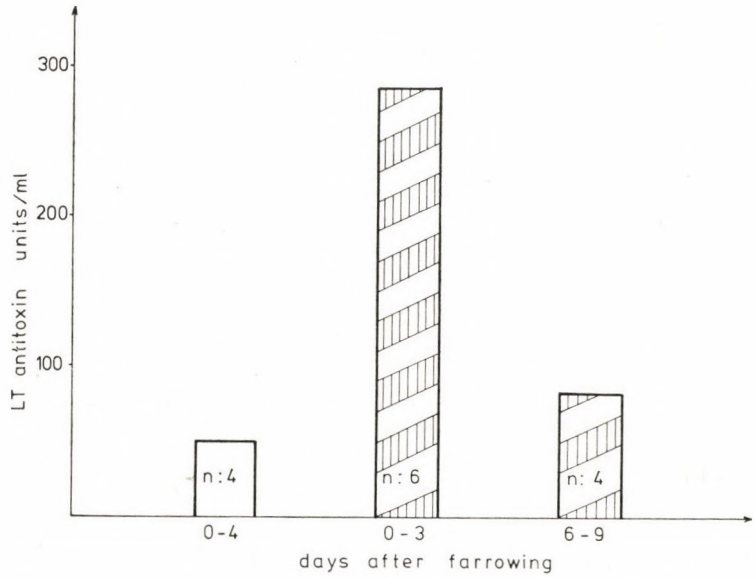


Fig. 3. LT-antitoxic titres incolostral whey of vaccinated and non-vaccinated sows
□ non-vaccinees; ▨ vaccinees

units/ml at 0 to 3 days after farrowing, indicating a considerable titre rise — almost sixfold relative to the control value — in consequence of the vaccination. The whey samples examined 6—9 days after farrowing contained, on the average, 84 LT antitoxin units/ml, still somewhat higher than the corresponding control level.

The vaccination induced a marked rise in the LT antitoxic level also in the colostrum. The whey level corresponded to about one half of the serum level in non-vaccinees.

Conclusions

Determinations of LT antitoxic levels in serum and colostral whey samples have unequivocally indicated that immunization of sows with our complex, enterotoxin containing *E. coli* vaccine induced considerable immune responses to the thermolabile enterotoxin of *E. coli*.

The titration procedures employed in this study proved to be suitable for the precise assessment of LT-antitoxic levels.

Adaptation of KASAI and BURROWS' (1966) procedure for determination of mean titres in paired serum samples (simplified procedure), as described in this report, seems to be a reliable approach to vaccination appraisal, and can also be used for determination of LT antitoxic titre in colostral whey.

In titre determinations based on the neutralization coefficient, incompleteness or absence of protection was also taken into consideration. The great advantage of the latter method is the exactness of the results, while it has the disadvantage of requiring a considerable number of experimental animals.

LT-neutralizing antibodies had already been present before vaccination. WILSON et al. (1971) demonstrated experimentally depression of LT-effect by sera of non-vaccinated sows, but did not pursue the quantitative relations of the antibody. LT-neutralizing effect of non-vaccinee sera was also reported by others (GYLES, 1974; WHIPP and DONTA, 1976; DOBRESCU and HUYGELEN, 1976), but information has generally been scanty on the LT-antitoxic levels normally found in pig sera at different ages and under large management systems. Our own experimental observations have suggested a gradual rise in the LT antitoxic titre with progressing age. This circumstance supports the presumption that the pigs used as serum donors in different farms of origin had either lived in an environment contaminated by *E. coli* strains, or had themselves been chronic carriers of such strains, or else, the relatively high anti-LT titres found were inexplicable.

The present findings permit the conclusion that the promising results obtained in the field trial of our *E. coli* vaccine were due primarily to the increase of anti-enterotoxic immunity. The 5- to 6-fold rise in the LT antitoxic titre over controls, as found not only in the serum, but also in the colostral whey of the vaccinees, weighs in favour of this conclusion. It should be noted that we did find LT-neutralizing antibodies also in the colostrum of non-vaccinees, unlike WILSON et al. (1971), who found none, probably because their methodical approach had not been sensitive enough for detection of minor levels. Since the LT enterotoxins produced by serologically different types of enterotoxic *E. coli* strains can be regarded as antigenically identical, there is reason to postulate that the immunization procedure developed by us can be protective not only against homologous, but also against heterologous *E. coli* infections. The fact that a minor proportion of the offspring of sows vaccinated during the field trial (PESTI and SEMJÉN, 1980) had developed *E. coli* diarrhea, suggests either a prenatal damage or the aetiological collaboration of hitherto indefined agents (Rotaviruses, etc.) in such cases.

Vaccination was followed by a considerable rise in the serum level of agglutinins to homologous strains. This was predictable, for the vaccine preparation used contained among others formol-inactivated type 0141: K85ab,

K88ab and 0149: K91, K88ac *E. coli* organisms. Agglutinating antibodies to a heterologous strain with K88 antigen component rose to a relatively high level, and those to a homologous 0141 strain with K88 component to a minor degree. This would suggest a major quantitative increase in antibodies to K88 in the postvaccination samples, but the actual result was less relevant than expected: the increase in anti-K88 agglutinins did not exceed one logarithmic order.

A possible interrelation between the postvaccination rise in toxin-neutralizing and agglutinating antibodies was also pursued. The OK antibodies were removed from hyperimmune serum samples by absorption with live organisms, and the purified sera were assayed for LT-neutralizing effect. The removal of the OK antibodies had practically no influence on the LT-neutralizing potential.

Obviously, the vaccination caused a notable rise also in the agglutinating antibody levels. However, as shown by SMITH (1976), OK-sera can protect exclusively against the homologous strain. RUTTER et al. (1976) hold the view that antibodies to K88 antigen play an important role in the specific protection of neonatal pigs against *E. coli* diarrhea. Since the applied vaccine did not provoke a notable antibody response to the K88 antigen we believe that anti-enterotoxic immunity is the decisive element in the protection by vaccination.

Summary

The cholera antitoxin titration procedure proposed by KASAI and BURROWS was adapted by a slight modification for determination of *E. coli* LT antitoxin in the serum and colostral whey of sows. Pre- and postvaccination samples obtained from sows under a large pig production system in connection with the field trial of an *E. coli* vaccine, contained 229 and 1116 LT-antitoxic units/ml, respectively, indicating an average fivefold rise in the basic LT antibody level in consequence of active immunization. A hyperimmune serum prepared by serial treatment with the above vaccine contained 3528 LT-antitoxic units/ml. The LT-neutralizing antibody content of the colostral whey increased to 5—6-fold of the low level found in non-vaccinees. The serum LT antitoxic titre rose with progressing age. Removal of OK antibodes from the hyperimmune serum by immunoabsorption had no influence on the LT-neutralizing potential. The authors believe that anti-enterotoxic immunity is the main element in the vaccine's protective effect.

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ИЗОЛЯЦИЯ ВИРУСА БЕШЕНСТВА ИЗ ХОМЯКА ОБЫКНОВЕННОГО (CRICETIS CRITECIS) В ЧЕХОСЛОВАКИИ

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Вирус бешенства патогенен для всех видов теплокровных животных (KAPLAN, 1953, 1969; JOHNSON, 1959; TIERKEL, 1959) и экспериментально бешенство было вызвано практически у всех изучаемых видов млекопитающих (ANDREWES, 1964). Однако отдельные виды восприимчивы неодинаково. По данным IRVIN (1970) среди 19 отрядов млекопитающих в качестве источника вируса бешенства обладают значением только два отряда: **Carnivora** и **Chiroptera**; третий отряд — **Rodentia** — играет роль только в определенных условиях. Приматы (включая человека), **Artiodactyla, Perissodactyla** часто инфицируются случайно, но не являются источником рабической инфекции, поскольку они не способны в широкой мере поддерживать это заболевание. То-же самое можно сказать и за счет представителей других отрядов, у которых бешенство случайно зарегистрировано. Несмотря на то, что бешенство в основном поддерживается за счет представителей указанных двух отрядов, оно может встречаться у представителей других отрядов, что имеет и практическое значение. Бешенство у них обусловлено контактом с видами, поддерживающими инфекцию.

Несмотря на указыванные данные, вопросы источников рабической инфекции в природе нуждаются в более детальном изучении. Грызуны являются особого значения в экологии вируса бешенства. При этом надо учесть и возможность перорального пути заражения, а также трофические связи, поскольку грызуны представляют собой основную часть пищи плотоядных.

Материал и методика

Изоляция вируса

Изученный материал. В 1972—1973 гг. мы изучили на бешенство 283 тушек хомяка обыкновенного (Cricetus cricetus) во время градации вида в восточной Словакии (1971—1973). Места добычи указаны в табл. I. Часть животных была поймана в живом виде, часть найдена погибшей.

Вскрытием полученный материал (головной мозг, включая продолговатый мозг и подчелюстные слюнные железы) мы изучили методом прямой иммунофлюоресценции (МПИФ) и биопробой на мышах (мозг и слюнные железы).

Определение рабического антигена МПИФ. Из поперечного среза мозга на уровне гиппокампусов готовились 3 мазки-отпечатки. После их сушки на

Таблица I

Результаты определения рабического антигена методом прямой иммунофлуоресценции и изоляцией вируса бешенства из хомяка обыкновенного (Cricetus cricetus) в восточной Словакии в 1972 – 1973 гг.

Местность	Общее к-во исслед.	Мозг						Подчел. сл. железа		
		МПИФ			биопроба			биопроба		
		исслед.	полож.	отриц.	исслед.	полож.	отриц.	исслед.	полож.	отриц.
Zemplínska Branč	14	14	3	11	14	3	11	12	—	12
Trebišov (ферма)	35	35	—	35	35	—	35	33	—	33
Čaňa	140	132	2	130	132	—	132	116	—	116
Pavlovce	9	9	—	9	9	—	9	9	—	9
Kriovšská Liesková	24	22	—	22	22	—	22	21	—	21
Seňa	46	41	—	41	41	—	41	38	—	38
Haniska	15	13	—	13	13	—	13	13	—	13
Итого	283	266	5	261	266	3	263	242	—	242

Примечание: МПИФ, метод прямой иммунофлуоресценции

воздухе, фиксации над пламенем, красились они прямым методом (Dean, 1966; Селимов и сотр., 1964). Для окраски применялся отечественный конъюгат (Bioveta, Ivanovice na Hané) в рабочем разведении 1 : 16. Мазки просматривались под люминисцентным микроскопом МЛ-2.

Биопроба на мышах. Из мозга и слюнных желез готовилась 20%-ная взвесь на забуференном физиологическом растворе pH 7,4 при добавлении 2—10%-ов нормальной инактивированной лошадиной сыворотки. Суспенсии до применения в биопробе хранились при —60 °C.

Для изоляции вируса мы применяли инбредные подсосные мышата из коммерческого питомника, которых заражали в мозг. Для инокуляции брали надосадочную жидкость 10%-ной суспензии мозга или слюнных желез, обработанную антибиотиками (Koprowski, 1966). Половину мышиного помета (4—5 голов) заражали материалом из мозга хомяков, а другую — из слюнных желез.

За животными наблюдали в течение 35 дней. Погибшие мышата изучались МПИФ.

В 1972 г. указанным способом мы изучили и тушки 196 кротов (Talpa europaea), отловленных в восточной Словакии.

Определение изолированных штаммов.

Три нами изолированных вирусных штамма (обозначеных 30, 7E, 9E) в предварительных опытах нами идентифицированы. Для сравнения мы применили уличный штамм 1151 (изолированный из мозга лисицы) и штамм CVS. Для идентификации мы применяли следующие методы: определение рабичес-

кого антигена МПИФ, определение телец Бабеш-Негри методом торможения ПИФ, проба вируснейтрализации, определение индекса нейтрализации, результаты внутримозгового титрования на мышах веса 6—8 г, определение инкубационного периода.

Материал. В опытах мы пользовались суспенсией мозга морских свинок, инфицированных указанными штаммами в дозе 500 $MICLD_{50}$, убитых в стадии прострации.

Метод прямой иммунофлуоресценции. Подробности метода приводятся в предыдущей части работы.

Определение телец Бабеш—Негри. Из поперечных срезов мозга морских свинок на уровне гиппокампусов готовили по 3 мазка-отпечатка, которые красили по Селлерсу (Tierkel, 1966) или Муромцеву (Селимов с сотр., 1964).

Метод торможения ПИФ. В первой фазе реакции применяли гипериммунную антирабическую сыворотку кролика в разведении 1 : 5; в другой — антирабический конъюгат для ПИФ.

Определение индекса нейтрализации осуществлялось по рекомендации ВОЗ (Johnson, 1966) на мышах веса 6—8 г. За животными наблюдали 28 дней. Титр вируса вычисляли методом Reed и Muench (1938). Из разницы значения титров вируса в реакции с добавлением нормальной инактивированной лошадиной сыворотки и антирабического иммуноглобулина вычисляли индекс нейтрализации.

Результаты

В 1972, 1973 гг. из 7 местностей в восточной Словакии методом прямой иммунофлуоресценции и биопробой на подсосных мышатах мы изучили на бешенство 283 тушки хомяка обыкновенного. Для определения рабического антигена МПИФ, в том числе изучено 266 хомячьих мозгов и 242 пар подчелюстных слюнных желез биопробой для изоляции вируса.

Из общего количества 14 хомяков, отловленных или найденных мертвыми в Земплинской Бранчи, МПИФ рабический антиген в мозгу обнаружен в 3-х случаях. В этих 3-х случаях из мозга изолировали вирус. Инфицированные вирусом подсосные мышата погибали к 14—18-у дню. Изолированные штаммы мы обозначили 3О, 7Е, 9Е. Кроме этого МПИФ рабический антиген нами обнаружен в 2-х случаях из общего количества 132 исследованных тушек хомяка из деревни Чаня. Но биопробой на мышах в этих 2-х случаях вирус не удалось изолировать. Результат изучения всех 242 пар слюнных желез хомяков биопробой на подсосных мышатах был отрицательным.

Результаты этой части опытов приведены в таблице I. Аналогичным изучением 196 тушек крота обыкновенного (**Talpa europaea**), отловленных в восточной Словакии (Земпленские Градиште, Чаньа) получен отрицательный результат.

Три нами изолированных вирусных штамма, обозначенных 3О, 7Е, 9Е, в предварительных опытах мы идентифицировали. Для определения мы применяли следующие методы: определение рабического антигена МПИФ, образование телец Бабеш—Негри, торможение МПИФ, определение индекса нейтрализации, результаты внутримозгового титрования на мышах, формирование инкубационного периода. В этих исследованиях мы применяли мозг морских свинок, инфицированных указанными штаммами. Результаты приведены в таблице II.

Таблица II

Биологические свойства штаммов вируса бешенства, изолированных из хомяка обыкновенного (**Cricetus cricetus**)

Штамм, происхождение	МПИФ+	Тельца Бабеш—Негри	Торможение МПИФ	Индекс нейтрализации	log $MICLD_{50}$	Инкубационный период
3 О (Cricetus cricetus)	+	+	+	3,7	5,2	12
7 Е (Cricetus cricetus)	+	±	+	3,9	7,3	7
9 Е (Cricetus cricetus)	+	+	+	3,8	5,4	15
1151 (Vulpes vulpes)	+	+	+	3,7	5,1	15
CVS (Париж)	+	−	+	4,0	7,3	6

Примечение: ПМИФ, метод прямой иммунофлуоресценции

Методом прямой иммунофлуоресценции рабический антиген нами обнаруживался всегда в виде полиформных гранул или скоплений. В мазках-отпечатках из мозга морских свинок, инфицированных штаммом 7Е, преобладали мелкие гранулы, часто в виде пыли. В препаратах окрашенных по Селлерсу и Муромцеву обнаруживались тельца Бабеш—Негри. Включений мы не находили в мозгу животных, инфицированных штаммом CVS. Результат этих исследований у животнжых, инфицированных штаммом 7Е, был сомнительным. Результат торможенния МПИФ во всех случаях был положительным. Индекс нейтрализации у всех изученных штаммов был относительно высоким; достигал показателя около 4,0. Уже при первом пассаже эти 3 хомячьих штамма давали относительно высокие титры (при внутримозговом заражении примерно $10^{-5,0}$). Штамм 7Е отличался особенно высоким титром ($10^{-7,3}$), подобно штамму CVS. Инкубационный период при внутримозговом заражении мышей за исключением штамма 7Е (7 дней) был характерным для уличных штаммов (12—15 дней).

На основании этих данных все 3 штамма, изолированных из хомяков, отличающихся друг от друга собственными биологическими свойствами,

можно считать идентичными с вирусом бешенства. У штамма 7Е предполагаем высокую степень аттенуации. Более подробные данные о биологических свойствах этих 3-х штаммов вируса бешенства мы приводим в одной нашей раньшей работе (ALEXANDER с сотр., 1976).

Обсуждение

В доступной литературе за исключением неполных данных NIKOLISCH (1965) и неопубликованных таковых BESEDA (1972), изолировавшего вирус из хомяков в Словакии, мы не нашли данных о бешенстве хомяка обыкновенного.

В 1972—1973 гг. нами изучено на бешенство 283 тушки хомяка обыкновенного во время градации этого вида в восточной части Словакии. Более подробные данные об этой градации хомяка опубликованы одним среди соавторов данной работы (GRULICH, 1973, 1975). Поскольку по настоящее время детально не изучены роль грызунов в экологии вируса бешенства (IRVIN 1970, WHO 1973), роль градации хомяков в Словакии (GRULICH, 1973, 1975), возможности повторения такой обстановки, вопрос является актуальным. Особенно если учесть, что бешенство животных регистрируется практически на всей территории Словакии, главным образом у диких животных и то, чаще всего у лисиц, для которых грызуны являются главной составной частью пищи. На основании этих обстоятельств, а также возможности пероральной инфекции, можно предполагать, что хомяк обыкновенный играет роль в эпизоотологии бешенства особенно во время его градации.

Из общего числа 283 тушек хомяка обыкновенного МПИФ рабический антиген обнаружен нами в 5, но биопробой на подсосных мышатах изолированы только 3 вирусных штамма (из мозга). Из подчелюстных слюнных желез вирус не выделен. При предварительной идентификации (определение рабического антигена МПИФ, образование телец Бабеш—Негри, торможение МПИФ, определение индекса нейтрализации и др.) мы установили, что 3 нами изолированных штамма являются вирусом бешенства. Отличаются они друг от друга своими биологическими свойствами. Один из них (7Е) является сильно аттенуированным. Более подробные данные о биологических;свойствах этих 3-х штаммов приводятся нами в нашей другой работе (ALEXANDER с сотр., 1976).

Резюме

В 1972 – 1973 гг. из 7 местностей восточной части Словакии методом прямой иммунофлуоресценции (МПИФ) для определения рабического антигена и биопробой на подсосных мышатах мы изучили на бешенство 283 тушек хомяка обыкновенного (Cricetus cricetus). Биопробой мы изолировали 3 вирусных штамма (3О, 7Е, 9Е). Аналогичным исследованием 196 тушек крота обыкновенного (Talpa europaea) из 2-х местностей Словакии вирус бешенства изолировать не удалось.

Три нами изолированных вирусных штамма в предварительных опытах идентифицированы (определение рабического антигена МПИФ, образование телец Бабеш-Негри, торможение МПИФ, определение индекса нейтрализации и др.) как штаммы вируса бешенства.

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ПЕРОРАЛЬНАЯ ИНФЕКЦИЯ ВИРУСОМ БЕШЕНСТВА ЛИСИЦЫ И КОШКИ ДОМАШНЕЙ

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Бешенство среди диких животных чаще всего встречается у некоторых видов отряда Carnivora, особенно семейства Canidae; в европейских условиях главным образом среди лисиц (**Vulpes vulpes**). На основании некоторых экологических особенностей лисиц и вируса бешенства не исключения кроме укуса возможность заражения вирусом бешенства и другими путями. При этом надо иметь в виду случаи изоляции вируса бешенства от представителей отряда Rodentia (в т. ч. в Чехословакии), являющихся основной пищей лисиц.

В доступной литературе за исключением наших раньших работ (**Švrček и Vrtiak**, 1967; **Vrtiak и Švrček**, 1970; **Švrček и сотр.**, 1972, 1975; **Švrček**, 1975) и работы **Bell и Moor** (1971) мы не нашли данных о экспериментальной пероральной инфекции вирусом бешенства у хищников.

Поскольку многие вопросы патогенеза бешенства у лисиц — особенно пероральной рабической инфекции — еще не решены и перспективы пероральной антирабической иммунизации неизвестны, мы решили изучить этот вопрос.

Материал и методика

Опытные животные

В опытах мы пользовались 15 лисицами возраста 7 месяцев и 15 беспородными кошками приблизительно одинакового возраста. До включения в опыт лисиц и кошек пробой вируснейтрализации на мышах (**Atanasiu**, 1966) серологически их изучили на наличие антирабических антител. Результат был отрицательным. Подробности этих исследований приводятся в наших предыдущих работах (**Švrček** с сотр., 1975).

Вирус и методы инфекции животных

В опытах мы применили два нами выделенных уличных штамма вируса бешенства (LB-1, L-22). Штамм LB-1 изолировали из подчелюстной железы лисицы; штамм L-22 из подчелюстной железы невакцинированной собаки. Свойства штаммов, история пассажей приводятся в наших предыдущих работах (**Švrček** с сотр., 1975; **Švrček**, 1975).

Опытных животных мы разделили на 6 групп (I—VI) по 3—6 годов в каждой. Первую группу — 6 лисиц (№ 1—6) и третью группу — 6 кошек (№ 10—15) мы заражали перорально: каждой лисице и кошке скормили по 5 мозгов мышей, инфицированных уличным штаммом LB-1 (приблизительно 1 000 000 $MICLD_{50}$). Таким же образом заражались животные пятой группы — 6 лисиц (№ 19—24) и шестой группы — 6 кошек (№ 25—30); животным скормили по 5 мозгов мышей, инфицированных штаммом L-22. Вторую группу — 3 лисицы (№ 7—9) и четвертую группу — 3 кошки (№ 16—18) мы инфицировали внутримышечно (в жевательную мышцу, двухсторонне, в дозе 1000 $MICLD_{50}$ штамма LB-1).

За инфицированными животными мы наблюдали клинически в течение 90 дней. Животных, которые в течение наблюдения заболели, мы убивали в стадии паралича; часть животных погибла. Незаболевшие животные убивались на 90-й день. Полученный при вскрытии материал (части мозга, роговица) мы изучали прямым методом иммунофлуоресценции (Dean, 1966; Schneider, 1969). Кроме этого биопробой на мышах проводили сравнительное титрование вируса в мозгу и в подчелюстных слюнных железах (Koprowski, 1966). Каждым разведением органа или ткани заражили по пять мышей интрацеребрально.

Результаты

Результаты первой части работы, направленной на изучение восприимчивости лисицы и кошки к пероральной инфекции уличным штаммом вируса бешенства приведены в таблице I. В течение 90 дней в группах животных, инфицированных внутримышечно (группа II — лисицы 7, 8, 9; группа IV — кошки 16, 17 и 18) наблюдались клинические признаки бешенства. Инкубационный период у лисиц колебался в пределах 20—35 дней, у кошек — 20—27 дней. Среди животных 4-х групп (24 голов, в том числе 12 лисиц и 12 кошек), инфицированных перорально, заболело только 6 голов; 4 кошки и 2 лисицы (25%-ная заболеваемость). Инкубационный период у лисицы, инфицированной штаммом LB-1 (животное I/1), равнялся 34-м дням. У двух кошек, инфицированных тем же штаммом (животные III/10, 15), инкубационный период колебался в пределах 31—40 дней. У лисицы, инфицированной перорально штаммом L-22 (животное V/23), он продолжался 25 дней, а у двух кошек, инфицированных одинаково тем же штаммом (животные VI/26, 28), колебался он в пределах 40—54 дней. У всех клинически заболевших животных диагноз был уточнен обнаруживанием рабического антигена методом прямой иммунофлуоресценции (в мазках и отпечатках из головного мозга).

Результаты второй части работы, направленной на изучение распределения рабического антигена при пероральной инфекции в некоторых частях центральной нервной системы, в роговице методом прямой иммунофлуорес-

Таблица I

Воспримчивость лисицы (**Vulpes vulpes**) и кошки домашней к пероральной инфекции вирусом бешенства. Клинические формы бешенства и гибель животных после поедания рабического материала

Группа жив.	Вид жив, №	Способ инфекций	Штамм уличного вируса	Доза $MICLD_{50}$	К-во погибших/к-во инфицированных	Инкубационный период
I.	лисица 1—6	через рот	LB-1	1 000 000	1/6	34
II.	лисица 7—9	внутримышечно	LB-1	1 000	3/3	20—35
III.	кошка 10—15	через рот	LB-1	1 000 000	2/6	31—40
IV.	кошка 16—18	внутримышечно	LB-1	1 000	3/3	20—27
V.	лисица 19—24	через рот	L-22	1 000 000	1/6	25
VI.	кошка 25—30	через рот	L-22	1 000 000	2/6	40—54

Таблица II

Восприимчивость лисицы (**Vulves vulvas**) и кошки домашней и пероральной инфекции вирусом бешенства. Определение рабического антигена в ЦНС и роговице методом прямой иммунофлуоресценции. Титрование вируса в мозгу и в подчелюстных слюнных железах

Группа животных	Вид, номер	Определение рабического антигена ПМИФ титер вируса — lg $MICLD_{50}$							
		кора мозга	Аммон. рога	мозжечек	продолг. мозг	спин. мозг поясн. ч.	роговица	подчелюстная железа	головной мозг
I.	лисица 1	+	+	+	+	+	—	—	3,7
II.	лисица 7	+	+	+	+	+	+	3,1	4,0
	лисица 8	+	+	+	+	+	—	—	3,9
	лисица 9	+	+	+	+	+	+	3,0	4,2
III.	кошка 10	+	+	+	+	+	+	2,8	3,5
	кошка 15	+	+	+	+	+	+	2,9	4,0
IV.	кошка 16	+	+	+	+	+	+	3,5	4,0
	кошка 17	+	+	+	—	+	+	3,2	3,1
	кошка 18	+	+	+	+	—	—	2,7	3,4
V.	лисица 23	+	+	+	—	+	+	2,9	4,0
	кошка 26	+	+	+	—	+	+	3,1	3,7
	кошка 28	+	+	+	+	+	+	—	3,8

ценции и титрованием вируса в мозгу и в подчелюстных слюнных железах приведены в таблице II. Для сравнения приведены в таблице и результаты этих же исследований у лисиц и кошек, инфицированных внутримышечно.

У всех животных, инфицированных перорально и внутримышечно (убитых в стадии начинающегося паралича и погибших), рабический антиген мы определяли в коре полушарий, в Аммоновых рогах и в мозжечке. В одном случае в 12 исследованных животных рабический антиген мы не обнаружили в поясничном отделе спинного мозга (кошка IV/18, инфицированная внутримышечно штаммом LB-1). У 2-х кошек (животные IV/17 и IV/16, инфицированных через рот штаммом м L-22) и у одной лисицы (животное V/23, инфицированной перорально штаммом L-22) рабический антиген мы не обнаружили в продолговатом мозгу. Среди 12 изученных животных роговичная проба была положительной у 9 голов, причем отсутствие рабического антигена в роговице было в соответствии с отрицательным результатом биопробы из подчелюстных слюнных желез в 2 случаях (лисица I/1, инфицированная перорально штаммом LB-1 и лисица II/8, инфицированная внутримышечно штаммом LB-1), в одном случае (кошка IV/18, инфицированная внутримышечно штаммом LB-1) роговичная проба была отрицательной несмотря на то, что биопробой на мышах вирус мы обнаружили в слюнной железе. В одном случае (кошка VI/28, инфицированная перорально штаммом L-22) антиген мы обнаружили в роговице, но его не было в подчелюстной слюнной железе. Биопробой на мышах за исключением 1-ого случая (кошка IV/17, инфицированная внутримышечно штаммом LB-1) вирус обнаруживался во всех случаях. В 3 случаях вирус мы не находили в подчелюстной слюнной железе (лисица I/1, инфицированная перорально штаммом LB-1, лисица II/8, инфицированная внутримышечно штаммом LB-1 и кошка VI/28, инфицированная перорально штаммом L-22).

Обсуждение

Данные эксперименты являются продолжением наших предыдущих работ (Švrček и Vrtiak, 1967; Vrtiak и Švrček, 1970), когда мы после скармливания рабического материала лисицам не наблюдали у них клинической формы бешенства. Причиной этого явилось вероятно малое количество подопытных животных.

В экспериментах, результаты которых представлены в данной работе, у части перорально инфицированных лисиц и кошек мы наблюдали клиническую форму бешенства. Эта же форма болезни наблюдалась нами уже и раньше (Švrček и Vrtiak, 1967, Vrtiak; и Švrček, 1970). Из доступной литературы известны также данные Bell и Moor (1971), которые пероральной инфекцией вызывали клинику бешенства у скункса (**Mephitis mephitis**), но у хорька (**Mustela putorius**) и кошки они этого не наблюдали. Кроме случайных причин, это может быть следствием свойств штамма, который применялся в их опытах.

На основании данных литературы и результатов собственных исследо-

ваний считаем, что возможность нероральной рабической инфекции у плотоядных является экспериментально установленным фактом. Но из сравнения доз вируса для пероральной и парэнтеральной инфекцийи, процента гибели вытекает, что для пероральной инфекции нужны очень высокие дозы высоковирулентного вирусного штамма и при таком пути заражения клинически заболевает относительно малое количество животных.

Вторая часть наших экспериментов была направлена на изучение распределения рабического антигена в центральной нервной системе, в роговице а также на сравнительное количественное изучение наличия вируса в мозгу и в подчелюстных слюнных железах.

Биопробой на мышах во всех случаях мы обнаруживали вирус в мозгу клинически заболевших животных и, за исключением одного случая (кошка, инфицированная интрамускулярно), всегда в более высоком титре, чем в слюнных железах. Это обстоятельство нами наблюдалось и в нашей предыдущей работе (Švrček с сотр., 1972). Согласующиеся результаты сообщены также Sikes (1962) от парэнтеральной инфекции. Это вероятно связано с высокой восприимчивостью лисиц и кошек. У части заболевших животных вирус в подчелюстной слюнной железе мы не обнаруживали. При сравнении результатов титрования вируса в слюнных железах и в головном мозгу у животных, инфицированных перорально и интрамускулярно, мы не наблюдали существенного различия.

Равным образом и сравнение результатов обнаруживаемости рабического антигена в центральной нервной системе и роговице животных, клинически болевших от пероральной или интрамускулярной инфекции, не показало существенных различий. Распределение рабического антигена, особенно в 3-й стадии инфекции (Schneider и Schoop, 1972), обладает большим диагностическим значением.

Lee и Becker (1972) наблюдали хорошие результаты в диагностике бешенства определением рабического антигена исследованием мазков-отпечатков из поясничного отдела спинного мозга. В наших опытах мы в одном случае (среди 12 исследованных) рабический антиген не обнаружили в спинном мозгу, но в других частях центральной нервной системы, за исключением продолговатого мозга, где отсутствовал, в 3 случаях он налицевствовал.

В виду того, что до настоящего времени не существовал надежный метод лабораторной прижизненной диагностики бешенства, роговичная проба, предложенная Schneider (1969), кажется перспективной. Beseda (1972) и Horyna и Kabelik (1973) тоже отмечают ее высокую диагностическую ценность. В наших экспериментах мы не всегда наблюдали соответствия между наличием рабического антигена в роговице и вируса в слюнной железе. Более подробные данные о распределении вируса бешенства у перорально и внутримышечно инфицированных животных (особенно в паренхиматозных органах) приводятся нами в другой нашей работе (Шврчек с сотр., 1975).

Резюме

В работе сообщается о первых результатах провоцирования клинической формы бешенства у лисицы обыкновенной (Vulpes vulpes) и кошки домашней путем заражения через рот и первом прижизненном определении рабического антигена в роговице плотоядных. Корнеальная проба и изучение мазков-отпечатков поясничного отдела спинного мозга прямым методом иммунфлуоресценции могут служить для уточнения диагностики бешенства (прижизненной или послесмертной).

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ЭКСПЕРИМЕНТАЛЬНЫЕ ПРЕДПОСЫЛКИ И ТЕХНИКА ПЕРОРАЛЬНОЙ АНТИРАБИЧЕСКОЙ ИММУНИЗАЦИИ ДИКИХ ПЛОТОЯДНЫХ. I. ПРЕДВАРИТЕЛЬНЫЕ ОПЫТЫ НА ЛАБОРАТОРНЫХ ЖИВОТНЫХ

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В настоящее время профилактика бешенства среди диких плотоядных и борьба с ним ограничивается резким понижением плотности населения хищников вообще, особенно лисиц. Сомнительна оправданность таких мероприятий в силу того, что они могут быть причиной нарушения равновесия в природе, дальше, их эффективность приспособительные явления (миграция, повышение приплода) могут тоже снизить.

Эти обсоятельства нас побудили к разработке принципиально нового метода профилактика бешенства у диких плотоядных — пероральной антирабической иммунизации. Первые результаты наших опытов, направленных на изучение пероральной антирабической иммунизации, мы сообщили в 1970 году (Вртяк и сотр.).

Материал и методика

1. Антигенная активность антирабических вакцин при пероральном введении. Опыты на морских свинках

Животные. В опытах мы применили 80 голов морских свинок веса 300—350 г из питомника **VELAZ Praha.** Животных мы разделили на 16 групп (I—XVI) по 5 голов в каждой. Животные отдельных групп помещались совместно в одной клетке.

Вакцины и иммунизация животных. В опытах нами применялись 8 разных антирабических вакцин.

Живая культуральная антирабическая вакцина из штамма Внуково-32 (лиофилизированная, серия № 13, выпущенная ИПВЭ* АМН СССР) (Аксенова и Селимов, 1969). Титер вируса до лиофилизации lg $10^{-5,0}$, после сушки

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$-10^{-2,5}$/0,3 мл. Результат определения иммуногенности в ИПВЭ: $ED_{50} = 5,6$ μl, индекс иммуногенности 1,1.

Живая культуральная антирабическая вакцина из штамма Внуково-32 (жидкая, приготовленная таким же образом как и первая, лиофилизированная). Инфекционный титер $10^{-4,5}$. Иммуногенность определена нами ($ED_{50} = 0,4$ μl).

Живая лиофилизированная (коммерческая) эмбриональная вакцина из штамма **Flury LEP (Vakcina proti vzteklině, avianizovaná)**, выпущенная **Bioveta (Ivanovice)**, серия № 133, титер вируса $10^{-3,0}$, $ED_{50} = 39,12$ mg.

Живая эмбриональная вакцина (жидкая) из штамма **Flury LEP**, приготовленная в нашей лаборатории размножения штамма **Flury LEP** по методу ВОЗ (**Koprowski, 1966**) на 7-дневных куриных эмбрионах. Из эмбрионов мы приготовили 30%-ную взвесь, провели титрацию на мышах ($10^{-4,9}$); титер мы потом привели до $10^{-4,5}$. Результат определения иммуногенности — $ED_{50} = 14,2$ mg.

Живая лиофилизированная (коммерская) эмбриональная вакцина из штамма **Flury HEP (Avianizovaná vakcina proti vzteklině Flury HEP)**, выпущенная **Bioveta (Ivanovice)**, серия № 14. Титер вируса — $10^{-2,7}$, $ED_{50} = 40$ mg.

Живая лиофилизированная культуральная вакцина из штамма **Flury HEP**, выпущена **VÚHEM (Praha)**. Титер лиофилизированной вакцины $10^{-3,5}$, $ED_{50} = 20,0$ μl.

Живая (коммерческая) мозговая лиофилизированная вакцина **Fermi (Vakcina proti vzteklině lyofilizovaná)**, выпущена **Bioveta (Ivanovice)**; серия № 0,38. Титер вируса $10^{-2,5}$, $ED_{50} = 5,7$ mg.

Инактивированная мозговая коммерческая вакцина **Hempt**, выпущена **ÚSOL (Praha)**, серия № 13, $ED_{50} = 8,24$ mg.

В нашей лаборатории определение иммуногенности вакцин мы осуществляли по методу ИПВЭ (**Selimov** и **Klujeva, 1979**) после нашей частичной модификации (**Švrček** и сотр., 1972).

Указанные виды вакцин мы вводили перорально в дозе 1,0 мл или подкожно в дозе 0,25 мл.

Определение антител. На 30-й и 60-й день после иммунизации мы определяли антирабические антитела пробой вируснейтрализации (ПВН) и методом косвенной иммунофлуоресценции (МКИФ). Антитела определялись только качественно при разведении сыворотки 1 : 4.

Проба вируснейтрализации. Проба вируснейтрализации осуществлялась на мышах по методу ВОЗ (**Atanasiu, 1966**). Конечное разведение — 1 : 4. В реакции применялся фиксированный штамм CVS в дозе 20—25 $MICLD_{50}$.

Метод косвенной иммунофлуоресценции. В качестве источника рабического антигена мы применяли мазки-отпечатки мышей веса 12—18 г,

инфицированных интрацеребрально фиксированным штаммом CVS. Мазки фиксировались аналитическим ацетоном при —20°C 24 часов. Изучаемые сыворотки разводились до 1 : 4 надосадочной жидкостью 20%-ной нормальной мозговой взвеси кролика. В реакции применялся антикавический конъюгат, выпущенный **ÚSOL Praha.**

2. Иммуногенная активность антирабических вакцин при пероральном введении

Опыты на морских свинках

Животные. Для опытов мы применили 33 головы морских свинок веса 300—500 г из питомника **VELAZ (Praha).** Животных мы разделили в 5 групп (I—V), из которых в 4-х группах было по 7 голов, а в V-й (контрольной) — 5 животных.

Вакцины и иммунизация животных. В наших опытах мы применяли две антирабических вакцины.

Жидкую, живую, культуральную вакцину из штамма Внуково-32; инф. титр $10^{-4,5}$, $\mathbf{ED_{50} = 0{,}4\ \mu l}$;

Жидкую, живую, эмбриональную вакцину из штамма **Flury LEP**; инф. титр $10^{-4,5}$, $\mathbf{ED_{50} = 14{,}2\ mg}$.

Более подробное описание указанных вакцин, методы их приготовления, титрации, определения иммуногенной активности, показаны в предыдущей, части работы.

Указанными вакцинами перорально (нанесением каплями на корень языка) в дозе 1,0 мл или подкожно (на внутренней поверхности бедра) в дозе 0,25 мл, мы иммунизировали групп I—IV. V-ая (контрольная) группа — не иммунизировалась.

Реинфекция. На 30-й день после вакцинации 4 экспериментальных группы (I—IV) и контрольных животных (группа V) перезаражали. Для реинфекции применяли уличный штамм вируса бешенства LB-1, изолированный из подчелюстных желез лисицы. Для реинфекции мы применили 3-й пассаж этого штамма в виде мозговой взвеси от мышей, инфицнрованных интрацеребрально. Для заражения опытных и контрольных морских свинок мы пользовались разведением мозговой суспензии 1 : 40 в соответствии с рекомендацией Копровского (1966) — на одно разведение более высокой концентрацией, которая обеспечивает 100%-ную гибель, что равняется приблизительно 6 $\mathbf{GpIMLD_{50}}$. Животных мы заражали внутримышечно, в бедро. За инфицированными животными мы наблюдали 30 дней. Мазки-отпечатки мозга павших, реинфицированных животных изучались по методу прямой иммунофлуоресценции для определения рабического антигена (**Habel, 1966**).

3. Антигенная активность вакцины из штамма Внуково-32 (живой, инактивированной) при пероральной иммунизации кроликов в зависимости от дозы и схемы иммунизации

Животные. В опытах применили 35 голов кроликов породы шиншила, веса в 2,0—2,5 кг. Животных мы разделили в 7 групп (I—VII), по 5 голов в каждой.

Вакцины и иммунизация животных. Для иммунизации кроликов мы применили жидкую культуральную антирабическую вакцину из штамма Внуково-32. Более подробное описание этой вакцины, методика приготовления, титрирование, определение иммуногенной активности показаны в предыдущей части работы. В опытах мы применили 2 разновидности этой вакцины: живую вакцину с инфекционным титром $10^{-4,5}$;

инактивированную вакцину, которую мы приготовили инактивированием упомянутой живой вакцины. К инактивации применялся бетапропиолактон в разведении 1 : 2500 в течение 2 часов, при температуре 36,5°C, по методу, рекомендованному ВОЗ для приготовления утиной эмбриональной вакцины. Определение иммуногенной активности не проводилось. Заражением мышей в мозг проверена ее неактивность.

Указанными вакцинами перорально (каплями на корень языка) или подкожно (на внутренней поверхности бедра) мы иммунизировали кроликов (по схеме, указанной на табл. III).

Определение антител. На 30-й и 60-й день после вакцинации у иммунизированных животных определялись антитела пробой вируснейтрализации (ПВН) и методом косвенной иммунофлуоресценции (МКИФ). Нами проводилось только качественное определение антител (разведение сыворотки — 1 : 4; подробности приводятся в предыдущей части работы).

Результаты

1. Антигенная активность антирабических вакцин при пероральном введении. Опыты на морских свинках

Результаты определения антирабических антител ПВН и МКИФ (качественное разведение сыворотки — 1 : 4) на 30-й день после иммунизации морских свинок приводятся в таблице I. Результаты показали, что у групп животных, иммунизированных подкожно 0,25 мл-ами вакцины, антитела на 30-й день обнаружены у всех животных за исключением таковых группы X, которые иммунизировались яичной вакциной из штамма **Flury HEP** и животных, иммунизированных вакциной **Hempt** (группа XVI). У животных этих двух групп антитела обнаружены в 60 или 80% случаев.

Таблица I

Пероральная антирабическая иммунизация Антигенная активность отдельных видов антирабических вакцин. Опыты на морских свинках. Результаты определения антител пробой вируснейтрализации и методом косвенной иммунофлуоресценции на 30 день после иммунизации[1]

Группа животных	Вид вакцины (вакц. штамм)	Титер вируса log $MICLD_{50}$/0,02	Доза в мл	Способ введения	Определение антител[2]	
					ПВН (%)[3]	МКИФ (%)[4]
I.	Vnukovo-32 культ. лиофил.	2,5	1,0	p. o.	2/5 (40)	2/5 (40)
II.	Vnukovo-32 культ. лиофил.	2,5	0,25	s. c.	5/5 (100)	5/5 (100)
III.	Vnukovo-32 культ. жидк.	4,5	1,0	p. o.	4/5 (80)	4/5 (80)
IV.	Vnukovo-32 культ. жидк.	4,5	0,25	s. c.	5/5 (100)	5/5 (100)
V.	Flury LEP эмбр. лиофил.	3,0	1,0	p. o.	1/5 (20)	1/5 (20)
VI.	Flury LEP эмбр. лиофил.	3,0	0,25	s. c.	5/5 (100)	4/5 (80)
VII.	Flury LEP эмбр. жидк.	4,5	1,0	p. o.	1/5 (20)	1/5 (20)
VIII.	Flury LEP эмбр. жидк.	4,5	0,25	s. c.	5/5 (100)	5/5 (100)
IX.	Flury HEP эмбр. лиофил.	2,7	1,0	p. o.	0/5 (0)	0/5 (0)
X.	Flury HEP эмбр. лиофил.	2,7	0,25	s. c.	3/5 (60)	2/5 (40)
XI.	Flury HEP культ. лиофил.	3,5	1,0	p. o.	0/5 (0)	0/5 (0)
XII.	Flury HEP культ. лиофил.	3,5	0,25	s. c.	5/5 (100)	4/5 (80)
XIII.	Fermi мозг. лиофил.	2,5	1,0	p. o.	0/5 (0)	0/5 (0)
XIV.	Fermi мозг. лиофил.	2,5	0,25	s. c.	5/5 (100)	4/5 (80)
XV.	Hempt мозг.	0	1,0	p. o.	0/5 (0)	0/5 (0)
XVI.	Hempt мозг.	0	0,25	s. c.	4/5 (80)	3/5 (60)

Примечание: 1, качественное (разведение сыворотки 1 : 4); 2, числитель — количество животных с антителами, знаменатель количество исследованных таковых; 3, проба вируснейтрализации; 4, метод косвенной иммунофлуоресценции

Но из общего количества 40 голов морских свинок, иммунизированных перорально одной из 8 разных антирабических вакцин в дозе 1,0 мл, антирабические антитела мы обнаружили только у 8 животных (20%). Лучшие результаты мы достигли у животных, иммунизированных культуральной антирабической вакциной из штамма Внуково-32. У животных, иммунизированных яичной вакциной из штамма **Flury LEP** антитела обнаруживались редко. Вакцины из штамма Flury HEP (яичная, культуральная) и мозговые таковые при пероральной иммунизации не были антигенно активными. Основным критерием антигенной активности считаем результаты ПВН.

2. Иммуногенная активность антирабических вакцин при пероральном введении. Опыты на морских свинках

В этих опытах, являющихся продолжением предыдущих (также на морских свинках), животных мы иммунизировали двумя видами антирабических вакцин (культуральной из штамма Внуково-32 и яичной **Flury LEP**),

причем титр вируса был одинаковым ($10^{-4'5}$). На 30-й день после вакцинации житовных мы заражали внутримышечно уличным штаммом LB-1 в дозе 6 $GpIMLD_{50}$. Результаты приведены в таблице II.

Таблица II

Пероральная антирабическая иммунизация. Иммуногенная активность культуральной антирабической вакцины из штамма **Vnukovo-32** и эмбриональной вакцины из штамма **Flury LEP**. Опыты на морских свинках. Результаты реинфекции животных уличным штаммом вируса дешенства на 30-й день после иммунизации

Группа животных	Вид вакцины, вакц. штамм	Титер вируса log $MICLD_{50}$/0,02	Доза в мл	Способ введения	Доза реинфекции $GpIMLD_{50}$	Результат реинфекции	
						инкуб. пер.	смертность+(%)
I.	Vnukovo-32-культ.	4,5	1,0	p.o.	6	16—18	2/7 (28)
II.	Vnukovo-32-культ.	4,5	0,25	s.c.	6	0	0/7 (0)
III.	Flury LEP-эмбр.	4,5	1,0	p.o.	6	12—17	6/7 (86)
IV.	Flury LEP-эмбр.	4,5	0,25	s.c.	6	18	1/7 (14)
V.	—	—	—	—	6	11—16	5/5 (100)

Применение: + числитель — количество погибших, знаменатель — к-во инфицированных животных

В контрольной неиммунизированной группе мы наблюдали 100% гибель животных. Все подкожно иммунизированные вакциной Внуково 32 животные выжили, а среди иммунизированных яичной вакциной — выжило 86%. В группе животных, привитых перорально вакциной Внуково-32, выжило 5 голов (72%); а среди привитых яичной вакциной **Flury LEP**—только одна голова (14%).

3. Антигенная активность вакцины из штамма Внуково-32 (живая, инактивированная) в зависимости от дозы, схемы иммунизации при пероральной иммунизации кроликов

У животных, иммунизированных живой или инактивированной вакциной БПЛ из штамма Внуково-32 (различной дозой, применением разных схем), на 30-й и 60-й день после иммунизации мы обнаруживали антитела. Результаты приведены в таблице III.

Антитела к 30-у и 60-у дням мы определяли у всех животных, иммунизированных подкожно дозой 1,0 мл (живой и инактивированной вакциной). При пероральной иммунизации инактивированной вакциной антитела не обнаруживались.

У кроликов, иммунизированных живой вакциной в дозе 1,0 мл перорально (группа II) ПВН дала положительный результат на 30-й день в 40%-ах случаев и на 60-й день — в 20%-ах случаев. При повышении дозы до 3,0 мл (группа V), введенной однократно, антитела мы обнаруживали у 80%-ов

Таблица III

Пероральная антирабическая иммунизация. Влияние дозы вакцины, живой и инактивированной, схемы иммунизации у кроликов, иммунизированных культуральной вакциной из штамма Внуково-32. Результаты определения антител пробой вируснейтрализации (ПВН) и методом косвенной иммунофлуоресценции (МКИФ) на 30-й и 60-й день после вакцинации[1]

Группа животных	Вакцина	Титр $\log MICLD_{50}$	Доза в мл	Схема (дни)	Метод. введ.	Определение антител[2]			
						30. день ПВН (%)	МКИФ (%)	ПВН (%)	60. день КМИФ (%)
I.	Vnukovo-32-жив.	4,5	1,0	1.	s. c.	5/5 (100)	4/5 (80)	5/5 (100)	4/5 (80)
II.	Vnukovo-32-жив.	4,5	1,0	1.	p. o.	3/5 (60)	2/5 (40)	1/5 (20)	0/5 (0)
III.	Vnukovo-32-жив.	4,5	3,0	1., 2., 3.	p. o.	5/5 (100)	3/5 (60)	4/5 (80)	3/5 (60)
IV.	Vnukovo-32-жив.	4,5	3,0	1., 7., 14.	p. o.	5/5 (100)	4/5 (80)	4/5 (80)	3/5 (60)
V.	Vnukovo-32-жив.	4,5	3,0	1.	p. o.	4/5 (80)	4/5 (80)	4/5 (80)	4/5 (80)
VI.	Vnukovo-32-инакт.	0	1,0	1.	p. c.	5/5 (100)	5/5 (100)	5/5 (100)	5/5 (100)
VII.	Vnukovo-32-инакт.	0	3,0	1.	p. o.	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)

Примечание: 1, качественное определение (разведение сыворотки 1 : 4); 2, числитель — количество животных с антителами, знаменатель — количество исследованных таковых

животных на 30-й и 60й день. Лучшие результаты получены, если дозу 3,0 мл разделили на 3 партии. При этом не оказала влияния продолжительность интервалов между вакцинациями (день или неделя). У всех так привитых животных на 30-й день мы выявляли антитела; на 60-й день даже у 80%-ов привитых таковых.

Обсуждение

До настоящего времени нет надежного метода профилактики бешенства у диких плотоядных. Борьба с ним ограничена применением мероприятий, ведущих к резкому понижению плотности диких хищников, особенно лисиц. Несмотря на определенную эффективность этого мероприятия, надо учесть также его недостатки (понижение плотности населения лисиц — это временное явление, нарушение равновесия в природе и др.).

Эти обстоятельства нас побудили к разработке принципиально нового метода профилактики бешенства у диких плотоядных — пероральной антирабической иммунизации. Первые результаты наших опытов, направленных на изучение пероральной антирабической иммунизации, мы сообщили в 1970 году (Вртяк и сотр.).

В предварительном опыте на морских свинках, направленном на изучение антигенной активности разных видов антирабических вакцин при пероральном введении, лучших результатов мы добились применением живой культурной вакцины из штамма Внуково-32. Особенно среди животных, которым вводилась эта вакница с более высоким титром живого вируса. Очень низкой антигенной активностью отличалась вакцина из штамма **Flury LEP** (эмбриональная). Вакцины из штамма **Flury LEP** (культуральная и эмбриональная), мозговые вакцины (живые и инактивированные) не были антигенно активными при пероральном введении.

Аналогичные результаты в опытах на лабораторных животных и лисицах добились Селимов (1971), Селимов и сотр. (1972), **Debbie** и сотр. (1972).

В следующих опытах (тоже на морских свинках), направленных на изучение иммуногенной активности двух живых вакцин (культуральной из штамма Внуково-32 и эмбриональной **Flury LEP**) с одинаковым инфекционным титром ($10^{-4,5}$), мы получили одинаковые результаты, как и при изучении антигенной активности разных вакцин при пероральной иммунизации. У группы животных, вакцинированных перорально культуральной вакциной из штамма Внуково-32, мы наблюдали иммунитет у 72%-ов привитых животных. Вакцина из штамма **Flury LEP** при пероральном введении оказалась слабой иммуногенной способности.

В опытах на кроликах, иммунизированных перорально, инактивированная культуральная вакцина из штамма Внуково-32 была антигенно неактивной. Аналогичных результатов добились и **Debbie** и сотр. (1972). На основа-

нии наших результатов и литературных данных можно предполагать, что аттенуированные варианты штамма SAD/ERA Внуково-32, а также WIRAB, который применялся MAYER с сотр. (1972), являются перспективными для приготовления живой вакцины для пероральной иммунизации.

Опыты на кроликах проводились ради разработки схемы пероральной иммунизации и определения дозы вакцины. Повышение дозы живой культуральной вакцины из штамма Внуково-32 с 1,0 до 3,0 мл положительно отразилось на ее антигенной активности особенно, если вводили ее в три приема. Согласно MAYER и сотр. (1972) при пероральной иммунизации может налицевствовать и вторичный иммунологический ответ.

Резюме

В опытах на лабораторных животных (морские свинки, кролики) разработаны экспериментальные предпосылки пероральной антирабической иммунизации. Первые результаты изучения этого оригинального метода профилактики бешенства диких плотоядных авторы сообщили в 1970 году (Вртяк с сотр.).

При пероральной иммунизации морских свинок самой высокой антигенной активностью отличалась живая культуральная вакцина из штамма Внуково-32. Аналогичные результаты были достигнуты при изучении ее иммуногенной активности. В опытах на кроликах инактивированная вакцина из штамма Внуково-32 не была антигенно активной. Высоко аттенуированный вакцинный штамм Внуково-32 является перспективным для приготовления вакцины пероральной иммунизации.

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МОДИФИКАЦИЯ КОЛИЧЕСТВЕННОГО МЕТОДА ИПВЗ* ДЛЯ ОПРЕДЕЛЕНИЯ ИММУНОГЕННОЙ АКТИВНОСТИ АНТИРАБИЧЕСКИХ ВАКЦИН В ОПЫТЕ НА МЫШАХ

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Кроме безвредности для реципиента, решающим значением обладает иммуногенность вакцин вообще, а антирабических в частности. Известно также, что применение стандартного метода изготовления вакцины не всегда гарантирует получение препарата, обладающего стойкой, высокой иммуногенностью.

В производстве очень трудно оценить иммуногенность антирабических вакцин на основании результатов их испытания на тех видах животных, для которых они предназначены. Для этой цели в настоящее время разработаны методы определения иммуногенной активности антирабических вакцин в опыте на лабораторных животных. Некоторые из них рекомендуются и Всемирной организацией здравоохранения WHO (1973), так например Habel (1973), Seligman (1973) и Koprowski (1973). В ЧССР для определения иммуногенности антирабических вакцин для животных (Šnoflák с сотр., 1967, Čupera, 1969) применяется метод Государственного научно-контрольного института (Назаров, 1961).

В нашей лаборатории успешно применяем метод Института полиомиэлита и вирусных энцефалитов АМН СССР (Клюева и Селимов, 1968; Selimov and Klujeva, 1969), который мы модифицировали (Švrček с сотр., 1972; Švrček с сотр., 1973; Vrtiak с сотр., 1973; Švrček с сотр., 1975).

Материал и методика

Разведение вакцин. Мозговые вакцины разбавить бидистиллированной водой до 5%-ной концентрации мозгового вещества; эмбриональные — до 33%-ной эмбриональной ткани; лиофилизированные тканевые вакцины до исходного объема. При этом исходим из предположения, что 5%-ная мозговая вакцина эквивалентна исходной, неразведенной инфекционной культуральной жидкости, содержащей вирус фикс типа Пастер; 33%-ная эмбриональная ткань эквивалентна неразведенной культуральной жидкости, содержащей авианизированный штамм (Флюри).

Дальнейшие нужные разбавления проводить по приложенной таблице; делать 5-кратные разбавления на физиологическом растворе.

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Примечание. Разбавлений следует делать такое количество, чтобы среднее разбавление обеспечило защищаемость 50%-ов иммунизированных мышей к реинфекции.

Иммунизация мышей. Вакцины в выше указыванных разбавлениях (см. табл. I) вводить мышам веса 12—14 г, подкожно, в области верхней губы, в объеме 0,2 мл, однократно. Каждым разбавлением иммунизировать 10—15 мышей. Количество введенной вакцины тканевой культуральной жидкости приведено в таблице I.

Таблица I

Схема разбавления вакцин; содержание вакцины в 0,2 мл в отдельных разведениях

Разведение	Вид вакцины			
	Мозговая	Культуральная из штамма т. Пастер	Эмбриональная	Культуральная из авианизированного штамма
основное	5 % = 10 mg	неразвед. = 10 μl	33 % = 66 mg	неразвед. = 66 μl
1 : 5	2 mg	2 μl	13,2 mg	13,2 μl
1 : 25	0,4 mg	0,4 μl	2,64 mg	2,64 μl
1 : 125	0,08 mg	0,08 μl	0,52 mg	0,52 μl
1 : 625	0,016 mg	0,016 μl	0,104 mg	0,104 μl
1 : 3125	0.0032 mg	0,0032 μl	0,02 mg	0,02 μl

Реинфекция мышей. Проводить на 21-й день после иммунизации. Для реинфекции применять уличный штамм или вирус фикс штамм CVS. Мышей инфицировать подкожно в другую сторону верхней губы. Для этого применить концентрацию на одно разведение выше, чем та, которая в предварительной титрации обеспечила 100%-ную гибель инфицированных мышей. Например, если уличный штамм вызывает 100%-ную гибель в разбавлении 1 : 80, то для реинфекции следует применять разбавление 1 : 40, или, если штамм CVS вызывает 100%-ную гибель при 1 : 2500, то для реинфекции брать — 1 : 500. За инфицированными мышами наблюдать 21 день.

До применения вируса для реинфекции провести его титрацию. Из основного разбавления (обычно 20%-ная мозговая суспензия на фосфатном буфере с добавлением 10% нормальной инактивированной лошадиной сыворотки)приготовить следующие разбавления (на забуференном физиологическом растворе с 2%-ами инактивированной лощадиной сыворотки): уличные штаммы — 1 : 10, 1 : 20, 1 : 40, 1 : 80, 1 : 160 и 1 : 320, штамм CVS — 1 : 500, 1 : 2500, 1 : 12 500 и 1 : 62 500. Каждым разбавлением инфицировать 5—10 мышей веса 16—18 г, подкожно в верхнюю губу, в объеме 0,1 мл. За мышами наблюдать 21 день.

Одновременно с реинфекцией иммунизированных мышей проводить контрольную титрацию вируса.

Инфективную дозу вакцины ($ЕД_{50}$), защищающую 50% мышей от последующей реинфекции, выраженную в милиграммах или в микролитрах (мозговой, эмбриональной ткани, или инфекционной культуральной жидкости) вычисляем по **Reed** и **Muench** (1938). Для этого применяем приложенные формулы (табл. II).

Таблица II

Формулы для вычисления $ЕД_{50}$ вакцины

1. % смертности $= \frac{100 \times \text{количество павших животных по кумул. данным}}{\text{сумма павших и выживших жив. по кумул. данным}} \times$

2. lg разведения 50% конечной точки $= \frac{\text{\% смертности выше 50} - 50}{\text{\% смертности выше 50} - \text{\% смертности ниже 50}}$

lg кратности разведения

3. $ЕД_{50}$ = аналог. разведения соотв. 50% конечной точке x количество вируссодержащей культ. жидкости (или мозговой, эмбр. ткани) введеной мышам в разведении, которое защищало менее чем 50% животных

Для оценки иммуногенной активности испытуемой вакцины в сравнении с референтной вакциной применяем индекс иммуногенности. Индекс определяем делением $ЕД_{50}$ референтной вакцины $ЕД_{50}$ испытуемой вакцины. Например, если $ЕД_{50}$ референтной вакцины равняется 0,6 мг-ам, а $ЕД_{50}$ испытуемой таковой равняется 0,25 мг-ам, то индекс иммуногенности будет 2,4.

Обсуждение

Применение стандартного метода изготовления вакцины не всегда гарантирует получение стандартного препарата вообще и антирабической вакцины в частности. В производственных условиях при серийном выпуске практически невозможно оценивать иммуногенность антирабических вакцин в опыте на тех видах животных, для которых они предназначены.

Широко применяемые методы определения уммуногенности антирабических вакцин в опыте на лабораторных животных, рекомендованные ВОЗ (1973), обладают некоторыми недостатками. Модифицированный метод **Habel** (1973) не является количественным. Оригинальный метод **Habel** (1973), как и таковой **Seligmann** (1973), количественные. Но при этих методах реинфекцию надо проводить интрацеребральным введением вируса. По данным **Habel** (1973) метод, применяемый для определения иммуногенности авианизированных вакцин (**Koprowski**, 1973), представляет собой неколичественный тест

на морских свинках. Его недостатком является и относительная дороговизна (морские свинки). Также метод ГНКИ (Назаров, 1961) является только полуколичественным тестом.

Метод ИПВЭ (Клюева и Селимов, 1968; SELIMOV and KLJUEVA, 1969) можно считать строго количественным, легко воспроизводимым тестом. В нем обеспечивается естественный периферический путь заражения. Нами предлагаемая модификация (ŠVRČEK и сотр., 1072; ŠVRČEK и сотр., 1973; VRTIAK и сотр., 1973; ŠVRČEK и сотр., 1975) заключается в универсальном, подкожном, однократном введении всех видов антирабических вакцин. Для реинфекции иммунизированных животных служит фиксированный вирус CVS.

Резюме

Предлагается модификация метода Института полиомиэлита и вирусных энцефалитов АМН СССР для определения иммуногенной активности антирабических вакцин в опыте на мышах. Метод является строго количественным, легко воспроизводимым и обеспечивает естественный периферический путь заражения иммунизированных животных. Модификация заключается в универсальном, подкожном, однократном введении всех видов антирабических вакцин. Для реинфекции иммунизированных животных служит фиксированный вирус CVS.

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PROPERTIES OF OVINE ADENOVIRUSES*

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In Hungary sheep production has been an important branch of livestock husbandry for several centuries. During the last decade its structure has been considerably altered by the establishment of large-production units for sheep and mutton lambs.

At present about 90% of the country's sheep stock is kept under large management systems. The stock was increased during the past few years. The altered conditions of production have been reflected chiefly in an increased mutton output. Recently 60—70% of the returns on sheep production have originated from marketing of mutton.

The changed conditions of management and, especially, of feeding, have expanded the range of veterinary problems in sheep husbandry. Concentration of stock has resulted in populations of inhomogeneous immunological history, in which the spread of known and unknown infectious diseases has been promoted by lack of protection in part of the animals.

Health problems also arose from random importations of scores of breeds for improvement of the indigenous Merino-type stock.

The altered conditions of husbandry and the various pathogenic agents introduced by the imported animals have contributed to the rising incidence of hitherto unknown or not yet encountered diseases in the sheep flocks since the years 1972 and 1973.

Mass incidence of such conditions prompted investigations into their aetiology. Many virus strains have been isolated from the nasal discharge and faeces of lambs during outbreaks of an acute disease. Evidence was obtained of the aetiological responsibility first of reoviruses, later of parainfluenza-3 virus and adenoviruses.

Epizootiological and virological investigations unequivocally indicated that adenoviruses accounted for the major part of the economic losses. In certain units 30—40% of the suckling lambs, and 10—15% of the fattening lambs died in outbreaks due to adenovirus infection. Major economic losses also arose from dinically inconspicuous chronic or latent forms of the infection, which could be identified only by careful clinical and virological examinations. Experimental evidence was presented that even clinically inapparent forms of adenovirus infection can severely affect the feed utilization by fattening lambs.

Isolation of adenoviruses from sheep have been reported from Northern Ireland (MCFERRAN et al., 1969, 1971), Scotland and Turkey (SHARP et al., 1974; BAUER et al., 1975). No unequivocal data were, however, presented on the pathogenicity of the isolates.

* This paper is an abstracted form of the author's thesis submitted under the same title to the Hungarian Academy of Sciences, in partial fulfilment of the requirements set to applicants for the postgraduate degree"Candidate of Science".

In the meantime, demand for an effective control was increasing in Hungary as the incidence of the adenovirus disease rose in the sheep flocks. Aetiological investigations were therefore started to obtain further information on the pathogenic properties of the viruses represented by the isolates. As up to the start of this programme in 1972, only two reports had been published in the literature on the isolation, and some proporties of ovine adenoviruses, many aspects remained to be approached by pioneer studies.

Virus isolations

Samples of nasal and pharyngeal discharge, faeces, urine, and organ homogenates, derived from 20 large sheep units, were tested, 647 altogether. In the overwhelming majority, embryonic ovine kidney (EOK) cell cultures were used for primary virus isolation. A hundred and twenty-eight adenovirus strains were isolated from lambs originating from 11 units. Isolates from the same outbreak always represented the same serotype.

The properties of the isolates were compared to those of the five known prototype strains of ovine adenovirus. Type-1 ovine adenovirus proved to be responsible for four outbreaks, while type-4 and type-5 each accounted for one. Further five outbreaks were due to adenoviruses not belonging to any known ovine serotype, but closely related to type-2 bovine adenovirus (BELÁK and PÁLFI, 1974a, b). This is remarkable, for as yet very little indication has been found of the natural spread of adenoviruses to a different host species or in other words, of the susceptibility of one species to closely-related adenoviruses of another.

The results of the isolation experiments and epizootiological observations supported the conclusion that the strains represented by the isolates played an aetiological role in outbreaks of respiratory and enteric diseases of sheep. The conditions of virus isolation and the results of examination of paired serum samples also weighed in favour of this implication.

Pathogenicity studies

Evidence of the pathogenicity of the isolates was obtained in successful infection experiments. Colostrum-deprived lambs developed symptoms similar to the natural disease on experimental infection with a type-5 isolate (strain PA/8). After a two-day latency period the lambs showed a serous nasal discharge, sneezing, conjunctivitis, lacrimation, diarrhoea, and a slight temperature rise. The postmortem and histopathological findings indicated that the virus infection gave rise to rhinitis and lobular pneumonia; the latter also

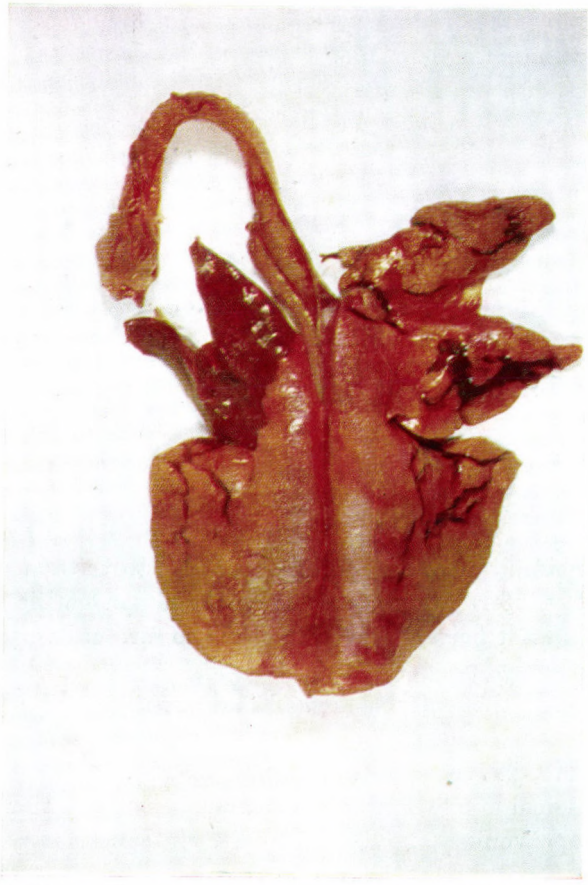

Fig. 1. Catarrhel pneumonia due to adenovirus infection in a lamb

occurred in naturally acquired cases (Fig. 1). Lesions in the liver and kidney showed that, in contrast to published observations, these organs, too, were affected.

Comparison of the pathogenic effects of strain PA/8 with those of the type-5 ovine adenovirus prototype strain (SAV) revealed that both gave rise to similar lesions in the respiratory and digestive tracts, but only PA/8 affected the kidney and the liver as well while the SAV strain, isolated from the faeces of clinically healthy animals, had no such effect. It was concluded that (a) both strains are pathogenic, but (b) despite the identity of their serotype, they are different in pathogenicity.

The information emerging from our infection experiments can be regarded as the first unequivocal evidence of the pathogenicity of ovine adenoviruses. It was also shown that the origin of the isolate and the conditions of its isola-

tion (from fresh outbreaks in the present cases) are more relevant in respect of pathogenicity than the serotype.

Recognition of the close relationship between certain ovine isolates and type-2 bovine adenovirus prompted us to infect lambs and calves with the former.

One type-2 bovine adenovirus-like ovine isolate (strain Het/3) equally caused disease in experimentally infected lambs and calves (BELÁK et al., 1975, 1977; TÚRY et al., 1975, 1978). The symptoms of the disease were similar in both species, and corresponded essentially to the condition elicited by other ovine adenoviruses. In this light, the prevailing concept of an almost absolute host specificity of adenoviruses seems to need revision.

Shedding of the virus was studied in great detail in the experimentally infected animals. Other investigators observed virus shedding exclusively with the faeces, while we isolated many strains also from the nasal discharge of acutely diseased lambs. Lambs infected experimentally by nasal and intratracheal instillation of virus material shed the agent copiously with both nasal discharge and faeces for 8—9 days after infection. Demonstration of renal lesions and simultaneous shedding of the virus with the urine has contributed to the scanty information available on the nephropathic effect of adenoviruses, which had not been observed in sheep so far, and very rarely in other species.

Evaluation of the epizootiological and experimental observations has led to description of the disease as the adenovirus pneumoenteritis of lambs (BELÁK and PÁLFI, 1974b; BELÁK et al., 1976a, b).

The aetiological analysis of the outbreaks has shown that the complicating conditions supervening on ovine adenovirus infections may be due, besides bacteria, to certain viruses. Of the latter, those affecting the same target organ(s) may aggravate the course of the disease. In addition to adenoviruses, the primary causal agents, reoviruses and parainfluenza-3 viruses were isolated as secondary agents from lambs involved in outbreaks of respiratory and enteric disease.

Physical and chemical properties of the ovine adenovirus isolates

In these studies, the five prototype strains of ovine adenovirus and the prototype strain (No. 19) of type-2 bovine adenovirus were used as reference strains.

The size and symmetry of the virions were established electron-microscopically, by negative contrast-staining technique. The virions were icosahedral, with an average diameter of 75 nm.

The buoyant density of the complete virions, centrifugation assessed by CsCl-gradient was 1.333 g/ml. A significant difference was found between

the behaviour of the bovine type-2-like ovine strains and bovine strain No. 19 on ultracentrifugation. The latter showed at least one band of incomplete virus particles while with the ovine strains at least three bands of these were separated. The differences found between the viruses of the two host species were consistently reproducible. It follows that the structure of the incomplete,

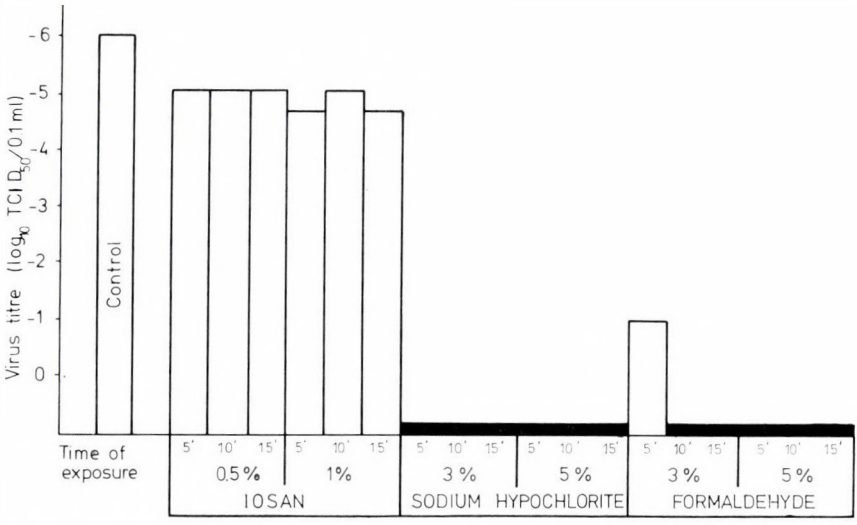

Fig. 2. Effect of different disinfectants on the infective titre of ovine adenovirus

defective virus particles may differ by host species also in the case of adenoviruses.

The ovine strains resisted 30-min exposure at 56 °C, although with a notable titre loss.

Incubation at pH 3.0, as well as chloroform and sodium deoxycholate treatment equally failed to depress the infective titre.

Resistance to common disinfectants was also tested. Sodium hypochlorite and formalin produced complete inactivation, while Iosan developed a much weaker action (Fig. 2).

The nucleic acid type was determined as DNA, from marked depression of infective titre during replication in the presence of 5-iodo-2'-deoxyuridine (IUDR).

The nucleic acid of the ovine viruses was also studied by the pronase-SDS-phenol technique in suspensions purified by gradient centrifugation, to obtain information on the genetic material, and to assess the degree of relationship between the antigenically related ovine and bovine strains by comparison of their DNA. The nucleic acid was isolated by ultracentrifugation, split

with restriction enzymes (Bam-HI, Sal-I and Eco-RI bacterial endonucleases), and the fragments thus obtained were separated by agarose-gel electrophoresis (Berencsi et al., 1978). The number, migration velocity and molecular weight of the fragments were determined. The electrophoretic properties of the intact nucleic acid isolated from adenoviruses of animals were in every respect consistent with those of the DNA isolated from human adenoviruses. The molecular weight of the ovine adenovirus DNA was determined as 23.3 megadaltons by summing up of the molecular weights of fragments.

A remarkable difference was found between the nucleic acids of the type-2 bovine adenovirus and the antigenically related ovine adenovirus strains. The DNA of the bovine prototype strain was split into fewer fragments by the restriction enzymes than did the ovine strains, and had an 5—6 megadaltons lower molecular weight compared to the latter. This accounted for a corresponding distinctness of the genetic material in the adenoviruses of the two host species.

The structural proteins of the virion were analysed by polyacrylamide-gel electrophoresis. The molecular weight of the hexon was 105 K; interestingly, lower in both ovine and bovine strains than in the human adenoviruses used as control.

Considerable differences were found between the structural proteins of the complete and incomplete virus particles separated by ultracentrifugation; certain protein components of the incomplete particles were lacking in the complete ones. This phenomenon has as yet been observed only in human adenoviruses.

Biological properties of ovine adenoviruses

The characteristics of the cytopathic effect (CPE) were unrelated to the serotype; certain strains caused a bunch-like aggregation of cells, while others a diffuse rounding or swelling.

Comparison of replication cycles in stationary and rotated cultures revealed considerable inter- and intra-typic differences, *e.g.* in the case of the ovine ORT/111 a strain related to the bovine adenovirus type-2, both the intra- and extracellular virus titre reached its peak by 40—46 hr. Against this, the type-1 ovine adenovirus strain, GY/14, had its titre maximum between 52 and 58 hr. Such differences have to be taken into consideration in vaccine production since. The replication rate was practically identical in stationary and rotated cultures, rotation seems not to be indispensable in ovine adenovirus isolation.

The course of the replication cycle did not notably vary with the age (0—7 days) of the embryonic sheep kidney cultures used. We suppose that the age of the cell culture plays little role between these limits.

Infected cell cultures were incubated at three different temperatures, 34, 37 and 40 °C. Each strain showed the highest replication rate at 40 °C, but strains of the same type behaved differently at the tested levels of incubation temperature.

Propagation of the ovine adenoviruses in various ovine and other mammalian cell cultures showed ready replication in lamb testicle, calf kidney, calf testicle, and pig kidney cells, and in a bovine cell line. Consequently, many cell lines can be used for the isolation and maintenance of ovine adenoviruses.

The CPE was studied in native and stained preparations by light microscopy, and in ultrathin sections by electron microscopy. Electron microscopically well definable virions appeared in the cultures about the 10th hr after infection.

Replication was also followed up by an indirect immunofluorescence technique developed by us. The fluorescence phenomena were of similar type to the direct test, although somewhat less pronounced. The viral antigen appeared in the nucleus of the host cell about 8 hr after infection. By 16 hr, about 90% of the cells had shown intracellular fluorescence. The test proved to be suitable for rapid aetiological diagnosis of adenovirus pneumoenteritis in lambs.

The ovine adenoviruses were tested for haemagglutinating activity in order to find out whether they have such a property at all, and whether, if they do haemagglutinate, their activity would be similar to that of the closely-related bovine adenoviruses. Among rat, monkey, human 0, bovine, ovine and chicken erythrocytes rat erythrocytes were agglutinated by all ovine adenoviruses. The bovine strains tested behaved similarly.

However, the ovine strains failed to agglutinate sheep erythrocytes, while the type-2 bovine prototype strain had a low agglutinating effect on these.

Still more remarkably, the ovine strains did not, while the type-2 bovine strain did, agglutinate calf erythrocytes. The specificity of this reaction was confirmed by the inhibition of the reaction by the antiserum to the type-2 bovine strain.

Ovine and bovine adenovirus strains equally failed to agglutinate *Macacus rhesus*, chicken, and human 0 erythrocytes.

On the basis of the haemagglutinating properties, the haemagglutination inhibition (HI) reaction was performed with rat erythrocytes. The HI test proved to be type-specific and its results showed a close correlation with those of the virus-neutralization test.

The haemagglutinating property of ovine adenoviruses was first demonstrated in this study. Typing is greatly facilitated by detection of antihaemagglutinin, a methodc onsiderably simpler and more less timeconsuming than the virus neutralization test.

Examination of the haemagglutinating properties made possible an unequivocal differentiation of serologically closely related ovine and bovine adenoviruses. The difference in their haemagglutinating behaviour appeared to be constant in the present studies, and can therefore be utilized as a simple and reliable intratypic differentiating marker of ovine and bovine strains.

Agar-gel diffusion and complement fixation (CF) tests presented evidence that all ovine strains possessed the common mastadenovirus antigen. Immunofluorescence tests have confirmed this finding.

The serum samples derived from sheep under large management systems contained precipitating antibodies which reacted exclusively with subgroup 2 bovine adenovirus antigen. This is suggestive of the existence a second subgroup of ovine adenoviruses. Antibodies to subgroup-2 bovine adenovirus were previously demonstrated exclusively in bovine serum; neither isolation experiments nor serological study have as yet disclosed the presence of this virus antigen in other species. It should be noted that the subgroup-2 bovine adenovirus strains fail to replicate in kidney cells; they were always isolated in testicle cell cultures, findings deserving consideration in the aetiological study of outbreaks.

Typing by virus-neutralization test revealed no cross-reactions between types or strains, and re-typing by HI test led to the same conclusion. Neutralization and HI tests equally presented evidence of a close antigenic relationship between certain ovine isolates and type-2 bovine adenovirus. It follows that at typing of an isolate not only the types known in the host species, but also those known in the entire Mastadenovirus genus should be taken into consideration.

The foregoing description of experimental observations on the ovine adenoviruses responsible for lamb pneumoenteritis has been centered on the aspects relevant to specific prevention. We have recently developed a bivalent vaccine from our own ovine adenovirus isolates (PÁLFI and BELÁK, 1978); this preparation confers a satisfactory passive protection the lambs of ewes immunized during pregnancy, and protects actively the mutton lambs until finishing when administered before transfer to the fattening unit. Application of the vaccine on this schedule has improved feed utilization even in lamb flocks in which the adenovirus infection was clinically inapparent.

The industrial-scale production of the vaccine is now in progress in the vaccine plant of the PHYLAXIA Veterinary Biologicals and Feedstuffs Co., Budapest. The details of preparation, and laboratory and field trials of the vaccine will be reported in another paper.

Summary

Reovirus, parainfluenza-3 and adenovirus strains were isolated from outbreaks of a respiratory-enteric disease affecting lamb flocks under large management systems. The adenovirus isolates were identified as the primary causal agent of lamb pneumoenteritis. Some

physical and chemical properties, of the adenovirus isolates as well as their resistance to heat, chloroform, deoxycholate and some disinfectants were determined. The virions contain DNA as genome. Also virion structural proteins were characterized. The cytopathic affects and the replication of the isolates were examined comparatively in stationary and rotated cell cultures of various origin and age. Replication was also followed up by electron microscopy and immunofluorescence. A haemagglutination marker differentiating the ovine isolates from the antigenically closely related type-2 bovine adenovirus is described. Precipitating antibodies reacting exclusively with subgroup-2 bovine adenovirus antigen were demonstrated in sheep sera. The detailed study of the properties of ovine adenoviruses has enabled us to develop specific prevention of adenovirus pneumoenteritis in lambs.

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DATA ON THE PROTEOLYTIC ENZYME SYSTEM OF *LERNAEA CYPRINACEA*

By

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Copepodes of the genus *Lernaea* are important pathogenic parasites of freshwater fish. In Hungary, occurrence of the species *L. cyprinacea* and *L. esocina* was already reported in the past century (Daday, 1918). *L. cyprinacea* was wide-spread in natural habitats at the middle of this century (Jaczó, 1950; Ponyi and Molnár, 1969), but it did not appear in pond farms until recently (Molnár, 1977). It was postulated by Podubnaya (1974) that the *Lernaea* species severely affecting cultured fish populations was not *L. cyprinacea*, but a morphologically indistinguishable parasite, *L. elegans*, which has been noted for a very broad host range.

The life cycle of *L. elegans* was studied in great detail by Nakai and Kokai (1931), that of *L. cyprinacea* by Lahav and Sarig (1964). Accordingly *Lernaea* females, after a short-term parasitic existence in the larval stage, establish themselves durably on the body surface of the fish hosts by digging their hooks and oral part deep into the latter's skin, from which they derive their nutriments. Khalifa and Post (1976) showed that haemorrhages and inflam matory reaction are developed at the sites of the parasites' attachment, but information ha-been lacking on the mode of intake and digestion of the host-derived nutriments.

The taxonomic problem whether the copepode parasite of cultured fish is *L. cyprinacea* or whether it is *L. elegans* remains to be resolved; we use the former designation. We studied the protein digestion of *L. cyprinacea* by analysis of its proteolytic enzyme system as reported in this paper.

Materials and methods

Parasites

Several hundreds of adult—*Lernaea* females, collected from the skin of a common carp (*Cyprinus carpio*) and a grasscarp (*Aristichthys nobilis*) host, kept in the same pond, were examined. The parasites were washed in distilled water three times, then homogenized in a Potter homogenizer for 10 min, in tenfold volume of distilled water. The homogenate was sonicated for 30 min at 1.2 A, and centrifuged for 15 min at 8000 r.p.m. The supernatant was used for further study. Preparation of the extract was carried out at +4 °C throughout, and the extract was stored in frozen state until used.

Enzyme activity assays

Proteolytic activities were measured on haemoglobin (Anson, 1938), casein (Rick, 1963) and gelatine (Kassai and Tóth, 1969) as substrate.

Spectrophotometric measurements were carried out in a Unicam SP 1800 Ultraviolet spectrophotometer, furnished with thermoregulated cuvettes, using a Unicam AR-25 Linear Recorder (Pye Unicam Ltd., Cambridge, England).

N-α-benzoyl-L-arginine ethyl ester hydrochloride (BAEE. BDH Chemicals Ltd., Poole, England) was used in 0.1 mM concentration, and absorption was read at 253 nm as proposed by SCHWERT and TAKENAKA (1955).

N-benzoyl-L-tyrosine ethyl ester (BTEE) (BDH) was used in 0.13 mM concentration, and the reaction was read at 256 nm (HUMMEL, 1959).

N-α-benzoyl-DL-arginine-4-nitroanilide-hydrochloride (BAPNA. Merck, Darmstadt, Germany) was used in 0.1 mM concentration. The assays were carried out as proposed by ERLANGER et al. (1961). The substrate was freshly prepared for each measurement (5.2 mg BAPNA was dissolved in 4 ml dimethyl-sulphoxide, and was made up to 10 ml with distilled water). Optical density (OD) was read at 380 nm.

N-α-toluene-4-sulphonyl-L-arginine methyl ester hydrochloride (TAME. Merck) was used in a concentration of 0.087 mM. HUMMEL's (1959) assay was applied; OD was read at 247 nm.

All enzyme assays were performed at 30 °C, in a system made up to 3 ml final volume, usually in 0.1 M TRIS—HCl buffer or, occasionally, in phosphate or borate buffer (see later).

Unit enzyme activity was defined as the activity incrasing the optical density of the system by 1.000 OD units in 1 min at 30 °C during the hydrolysis of the substrate, as read at the appropriate wave-length.

The following enzymes were used as reference preparations:

-trypsin from beef pancreas (EC 3.4.21.4.) (BDH).

-α-chymotrypsin from bovine pancreas (EC 3.4.21.1.) (BDH).

Determination of inhibitor activity

Lernaea extract was incubated for 10 min at 80 °C in order to inactivate the enzymic activity, a known amount of trypsin or chymotrypsin was added, and the enzyme activity was measured. As it was supposed that the parasite may contain heat-labile inhibitors, the test was also carried out in a native extract. Added and endogenous tryptic activities of the extract were both taken into consideration for the assessment of inhibitor activity.

Gel chromatography

Sephadex G-75 (Pharmacia Fine Chemicals AB, Uppsala, Sweden) was used for gel chromatography, after 3-hr treatment in 0.05 M TRIS—HCl buffer of pH 7.8 in boiling water, and storage for five subsequent days. The

column dimensions were 2.5 × 4 cm, and 0.05 M TRIS—HCl buffer of pH 7.8 was used as eluent. The void volume (V_0) was determined by passing Blue Dextran 2000 (Pharmacia) through the column; the total volume (V_t) was determined with $K_2Cr_2O_7$. The elution rate was 0.5 ml/min, from upside downward, and fractions of 5 ml volume were collected.

For the estimation of molecular weight (MW) the column was calibrated with materials of known MW. Each substance was chromatographed twice. The following substances were used as reference preparations, each in 15 mg amount: horse-heart cytochrome C, freeze-dried, salt free (Koch-Light Lab. Ltd., Colnbrook, England; MW 12 500) (TRISTRAM and SMITH, 1963)

-trypsin from beef pancreas (EC 3.4.21.4.) (BDH); MW 23 280 (BARMAN, 1969);

-ovalbaumin, twice crystallized (Calbiochem, San Diego, California, U.S.A.), MW 45 000 (ANDREWS, 1970);

-bovine serum albumin, fraction V,B. grade, cat 901683 (Calbiochem), MW 67 000 (ANDREWS, 1970).

The elution volume of the reference preparations was determined from the extinction values read at 280 nm. The relationship between elution volume and MW is characterized by the regression equation.

$$\lg MW = 4.9487 - 1.5902\ K_{av}$$

$$r^2 = 0.9985$$

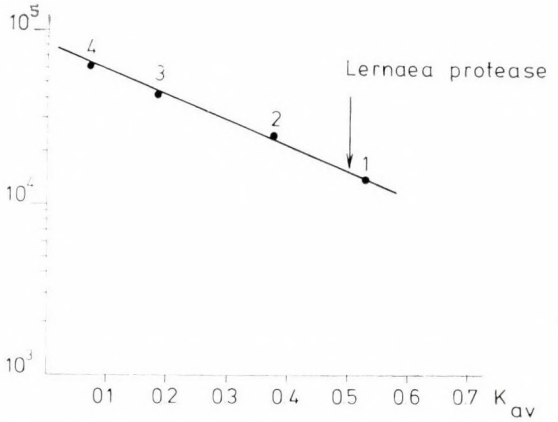

Fig. 1. Molecular weight of proteins versus K_{av}-values on Sephadex G-75 column

1. Cytochrome C MW: 12 250
2. Trypsin MW: 24 250
3. Ovalbumin MW: 45 000
4. BSA MW: 67 000

Regression: $\lg MW = 4.9487 - 1.5902\ K_{av}/r^2 = 0.9985$

which was calculated by means of a Hewlett Packard 9810 calculator. The MWs were estimated from the above equation. The calibration curve is shown in Fig. 1.

Examination of the effect of pH

The following buffer solutions were used: 1. Phosphate buffer (M/15 Na_2HPO_4 and M/15 KH_2PO_4); at values pH $>$ 8.0: M/15 Na_2HPO_4 + 0.5 M NaOH. 2. Borate buffer: 0.2 M sodium borate (12.404 g H_3BO_3 + 100 ml NaOH/l); the pH was adjusted up to 9.0 with 0.1 N HCl, above 9.0 with 0.1N NaOH. 3. TRIS buffer (for details, see "Enzyme assays").

Effectors

Three kinds of effectors were used:

1. Metal ion: $Ca^{2+}(CaCl_2)$
   $Mg^{2+}(MgCl_2)$
   $Zn^{2+}(ZnCl_2)$
   $Co^{2+}(CoCl_2)$
   $Mn^{2+}(MnCl)_2$

(All metal salts used were products of REANAL, Hungary).

2. Active group inhibitors:
   ethylenediaminetetra-acetic aci ddisodium salt (EDTA) (REANAL),
   p-chloromercurybenzoic acid (PCMBA) (BDH),
   1-chloro-3-tosylamido-7-amino-2-heptanone (TLCK), (SERVA),
   diisopropylfluorophosphate (DFP), (SERVA).
3. Natural protease inhibitors:
   Fish-serum trypsin inhibitor (STI),
   Soybean-trypsin inhibitor (SBTI), (BDH),
   Ascaris suum trypsin inhibitor (ATI) isolated by one of us (JUHÁSZ, 1979).

Results

Enzyme activity

The enzyme activity of the *Lernea cyprinacea* extract was assayed on haemoglobin, casein and gelatine as substrate. The extract splitted casein and haemoglobin strongly, and was less effective on gelatine. This was regarded as evidence of a proteolytic enzyme activity in *Lernaea cyprinacea* extracts.

The proteolytic activity was further assayed on the organic synthetic substrates BAEE, TAME, BAPNA and BTEE, and was found to be 3.19, 1.49, 1.12 and 0.00 units/g parasite, respectively, assessed as the mean for four measurements in different extracts. Thus, the extract had a distinct splitting effect on BAEE, TAME and BAPNA, but no effect whatever on BTEE, indicating that no chymotrypsin-like enzyme occurs in the *L. cyprinacea* extracts, its entire protease activity is tryptic in nature.

Inhibitor activity

The activities of known amounts of trypsin and chymotrypsin did not change after addition to the denatured extract, as assessed on either BAEE or BTEE as substrate.

Addition of α-chymotrypsin to the native extract did not change the activity of the latter. The joint tryptic activities of added bovine trypsin and native extract also remained unchanged. In view of this, presence of trypsin and chymotrypsin inhibitor(s) in the Lernaea extract can be ruled out with certainty.

Gel chromatography

The results of the chromatography of the Lernaea extract on Sephadex G-75 dextran gel column are shown in Fig. 2. Measurements of extinction at 280 nm showed maximum only in the lower molecular weight range, presumably in association with free amino acids, oligopeptides and other materials

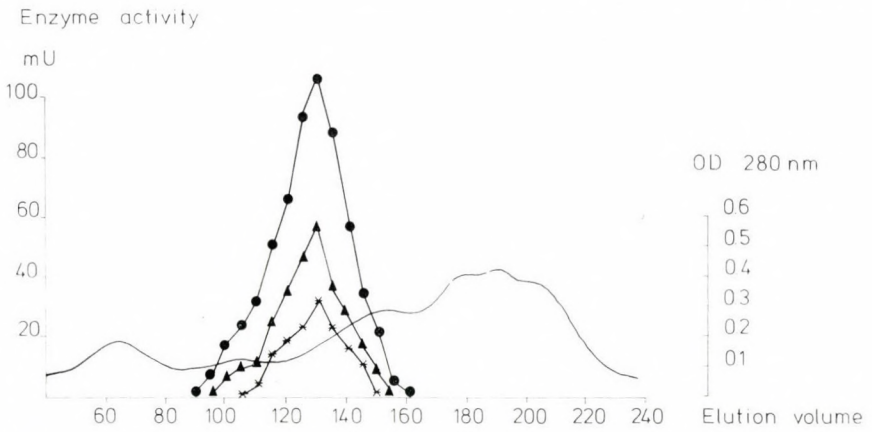

Fig. 2. Elution pattern of OD 280 and enzymatic activities of *Lernaea cyprinacea* extract on Sephadex G-75 column. Column dimensions: 2.5 × 42 cm. Eluent: 0.05 M TRIS—HCl (pH 7.8)

——— OD 280 nm
●——● BAEE-splitting activity
▲——▲ TAME-splitting sctivity
×——× BAPNA-splitting activity

having their absorption maxima at this particular wavelength. The enzyme activity was separated in a single symmetrical peak, at $K_{av} = 0.500$. Assays of the active fractions on different substrates (BAEE, TAME, BAPNA) yielded curves similar in every respect. The calculations indicated identical substrate-splitting activity at each point of the enzymatically active range, and the values found corresponded with the splitting activities determined in the original extract.

As the enzyme activity was associated with a single chromatographic fraction, and the splitting activity of the active fraction was identical with that assessed in the crude extract regardless of the kind of substrate used, there is reason to postulate that the detected activity represented a single enzyme.

The molar splitting activity of the enzyme, determined as μmol of substrate split by 1 g parasite in one min, was found to be 3.95, 2.76 and 0.127 on BAEE, TAME and BAPNA substrate, respectively (the corresponding $\Delta\varepsilon$ molar extinction coefficients were 0.808, 1.49 and 1.12). The computed values of the ratios of the molar splitting activities for BAEE/BAPNA and TAME/BAPNA were 31.09 and 21.72; thus the esterolytic splitting activity, regardless whether determined on BAEE or TAME, was always considerably higher than the amidase activity measured on BAPNA. The quotient of the peptide-bond-splitting activity of the substrates used for determination of esterolytic action (BAEE: TAME) was 1.43.

In further studies we used the active fractions separated by gel chromatography.

pH dependence

The *Lernaea* extract was tested for enzyme activity in three different buffer solutions. TRIS—HCl, phosphate and borate buffers were used. Below pH 5.0, no activity was demonstrable on BAEE substrate in any buffer and on BAPNA substrate in phosphate buffer. At pH 11.0, a correction factor was employed to make up for the decomposition of the substrate itself; above pH 11.0 the results were unreliable despite correction, and were therefore not included in the evaluation.

The pH dependence of the enzyme activity is shown in Fig. 3. The BAEE-splitting activity was highest in phosphate buffer, less pronounced in TRIS buffer and lowest in borate buffer. Maximum were found around pH 9.0 on BAEE in all three buffers and on BAPNA in phosphate buffer.

Effect of temperature

The temperature optimum was determined in TRIS buffer, both on BAEE and BAPNA. Maximum splitting activity was developed at 70 °C on both substrates. The conditions of the test are shown in Figs 4a and 5a.

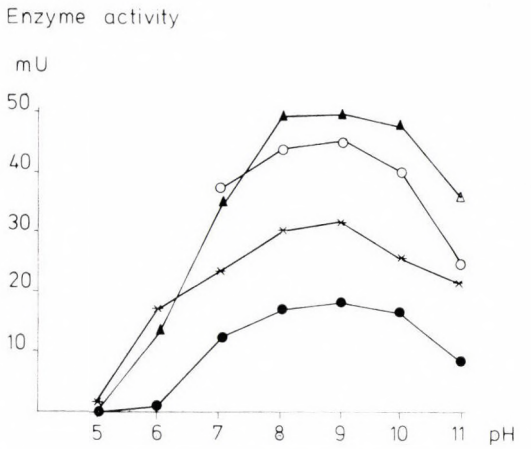

Fig. 3. Effect of pH and buffers on the proteolytic activity of *Lernaea cyprinacea* extract. The samples were assayed on BAEE in phosphate (▲——▲), TRIS (○——○) and borate (✳——✳), on BAPNA in phosphate (●——●)

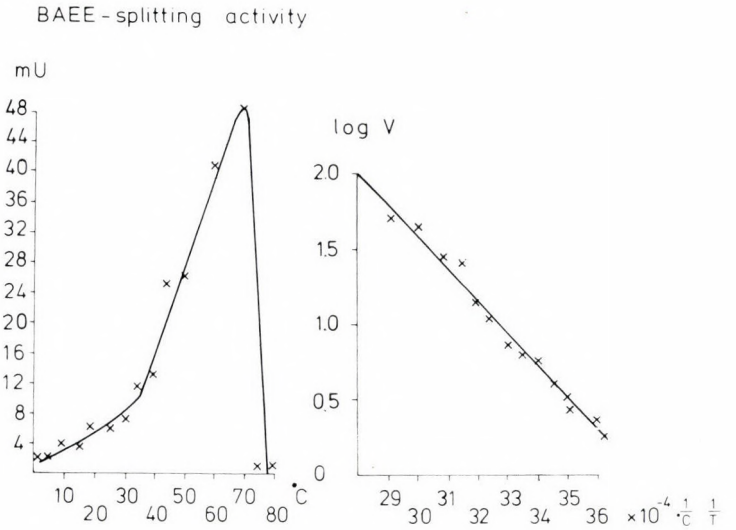

Fig. 4. Effect of temperature on the BAEE-splitting activity of *Lernaea cyprinacea* extract. Calculation of activation energy.

Regression: $y = 7.852 - 2090.616\,x (r^2 = 0.984)$

Determination of the temperature optimum made possible the calculation of the activation energy. The results are presented in Figs 4b and 5b.

The equation of regression characterizing BAEE-splitting activity in TRIS buffer was

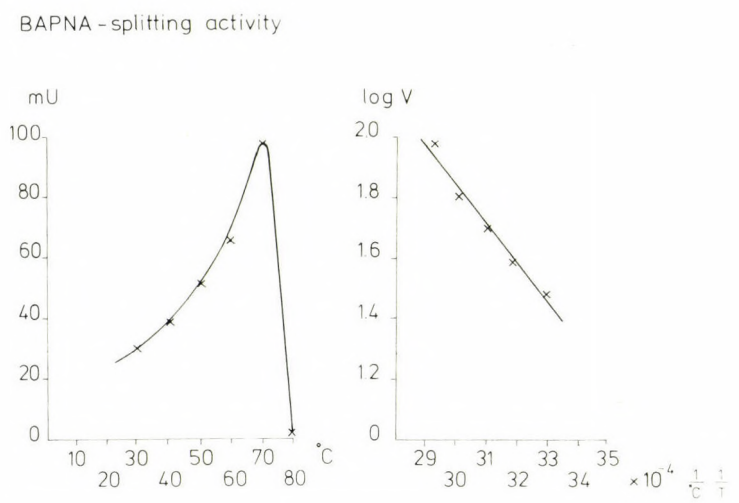

Fig. 5. Effect of temperature on the BAPNA-splitting activity of *Lernaea cyprinacea* extract. Calculation of activation energy

Regression: $y = 5.789 - 1314.183\,x (r^2 = 0.992)$

$$y = 7.852 - 2090 \cdot 616\,x$$

$$(r^2 = 0.9840)$$

From this, the activation energy was assessed as

$$E = -9561.97 \text{ cal}$$

$$(R = 1.986 \text{ cal} \cdot \text{degree}^{-1})$$

(Fig. 4b) by means of the Arrhenius equation

$$\text{tg}\,\alpha = \frac{-E}{2.302\,R}.$$

Similarly, the regression line for BAPNA splitting corresponded to

$$y = 5.789 - 1314.183\,x$$

$$(r^2 = 0.992)$$

at an activation energy of $E = -6010.9$ cal (Fig. 5b).

The thermostability of the enzyme, as determined on BAPNA at 70 °C, is shown in Fig. 6.

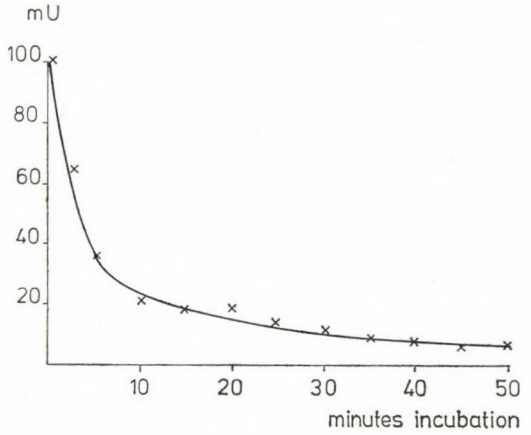

Fig. 6. Effect of temperature on the stability of *Lernaea cyprinacea* protease, as assessed on BAPNA

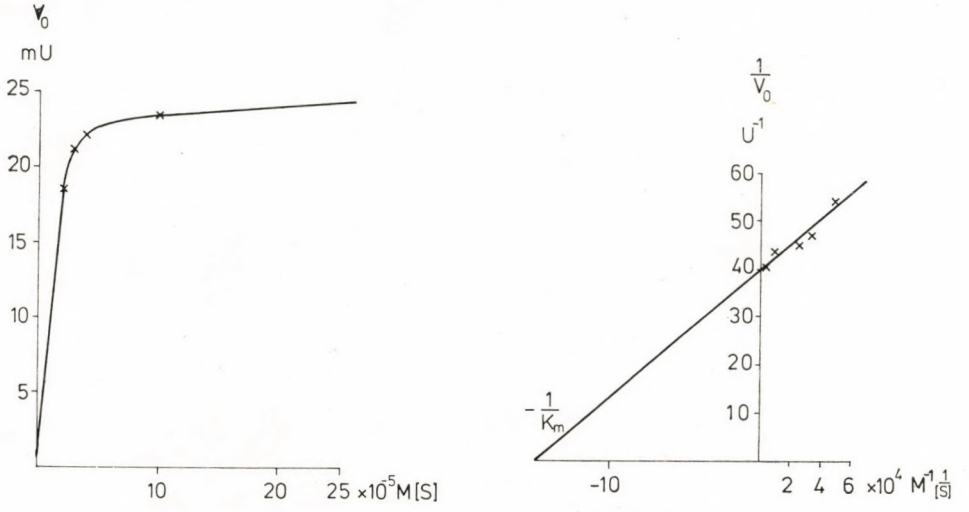

Fig. 7. Reaction rate versus substrate (BAEE) concentration. Calculation of Michaelis constant with the Lineweaver-Burk equation

Regression: $y = 39.468 - 2.669 \cdot 10^{-4}\,x (r^2 = 0.945)$

Michaelis constant

After assessment of the initial reaction rates (Fig. 7), the Michaelis constant was determined on BAEE substrate in TRIS buffer, by the Lineweaver-Burk equation, as

$$K_m = 6.8 \times 10^{-6}\,M.$$

The role of effectors

Bivalent cations were tested how to influence the proteolytic enzyme activity of *Lernaea cyprinacea*. The results are shown in Table 1.

Ca^{2+}, Mg^{2+} and Zn^{2+} had no influence on enzyme activity at 10^{-2} M concentration.

Table I

Effect of different activators and inhibitors on *Lernaea cyprinacea* protease and bovine trypsin

Enzyme	Effector	Concentration	Activation	Inhibition	Change in enzyme activity per cent
Lernaea protease	Ca^{2+}	10^{-3} M, 10^{-2} M	—	—	
1.6 mU BAEE	Mg^{2+}	10^{-3} M, 10^{-2} M	—	—	
splitting	Zn^{2+}	10^{-3} M, 10^{-2} M	—	—	
	Co^{2+}	10^{-3} M	+		+ 320
	Mn^{2+}	10^{-3} M	+		+ 190
		10^{-2} M	+		+1500
	EDTA	10^{-3} M		+	— 32
		10^{-2} M		+	— 100
	PCMBA	10^{-5} M		—	
	DFP	10^{-4} M		+	— 100
	TLCK	10^{-4} M		+	— 66
		10^{-3} M		+	— 100
Bovine trypsin 1.5 mU	Co^{2+}	10^{-3} M	+		+ 63
BAEE splitting	Mn^{2+}	10^{-3} M	+		+ 67
	EDTA	10^{-3} M		+	— 100

Co^{2+} and Mn^{2+} developed a measurable activating action. The 1500% activity increase elicited by 10^{-2} M Mn^{2+} was reversible with EDTA, which in fact depressed the enzyme activity completely.

EDTA developed a partial inhibitory action at 10^{-3} M, and a complete one at 10^{-2} M. Even 10^{-1} M Ca^{2+} failed to reverse the inhibition, but 10^{-3} M Co^{2+} did reverse it (and even increased the enzyme activity by 145%). Similarly, the inhibitory effect of EDTA was reversible with $2 \cdot 10^{-3}$ M Mn^{2+}.

Table 1 also includes data of inhibition by certain characteristic active groups. Since p-chloromercurybenzoic acid showed no indication of inhibitory action at 10^{-5} M concentration, the protease found cannot be regarded as a SH-protease.

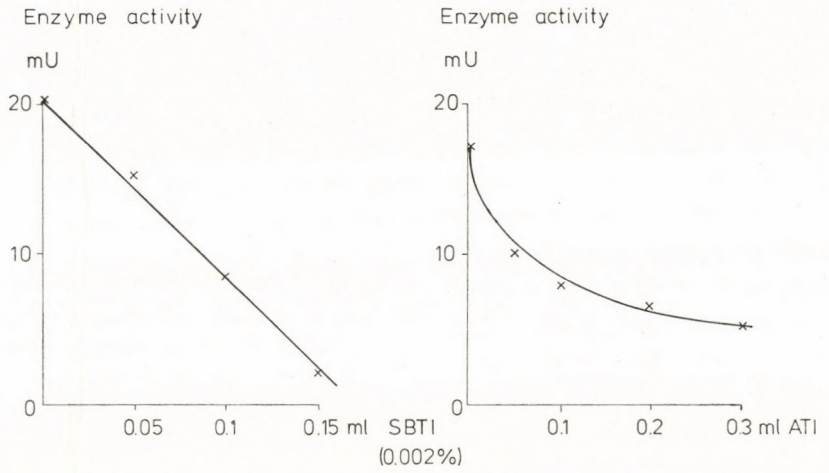

Fig. 8. Effect of rising inhibitor concentrations on BAEE-splitting activity of *Lernaea cyprinacea* extract.
SBTI: soya trypsin inhibitor; ATI: Ascaris suum trypsin inhibitor

10^{-4} M diisopropylfluorophosphate produced a complete inhibition after a 30-min pre-incubation. In view of this, the enzyme can be classified as a serine protease; depression of its activity by the specific trypsin inhibitor TLCK also supports this conclusion.

Finally, some natural protease inhibitors were tested for influence on the enzyme. Fish serum as a source of trypsin inhibitor failed to depress the enzyme activity, while STI and ATI did depress it very distinctly (Figs 8a and 8b).

It follows that the enzyme is sensitive to natural trypsin inhibitors, while its insensitivity to the serum trypsin inhibitor seems to be a functional necessity.

Bovine trypsin, too, was tested with some effectors, for sake of comparison (Table I); Co^{2+} and Mn^{2+} activated it at 10^{-3} M concentration, and the effect of both ions was reversible with 10^{-3} M EDTA. EDTA inhibited the activity of bovine trypsin at 10^{-3} M concentration. The inhibitory effect of DFP, TLCK, SBTI and ATI is an established fact.

The comparative examinations have indicated a striking similarity of the Lernaea protease to trypsin.

Discussion

The proteolytic enzyme systems of copepodes have chiefly been studied on the large-bodied decapodes; more recent investigations have been concerned with these and Amphipode species (Ponyi, 1966; Kleine and Ponyi, 1966;

PONYI et al., 1969). Digestive enzyme studies on *Lernaea cyprinacea* have been greatly hampered by minute body dimensions and relatively limited availability of this species.

The present findings have unequivocally affirmed that *Lernaea cyprinacea* possesses a powerful proteolytic enzyme activity, which resists the trypsin inhibitor taken up by the parasite from fish blood. Gel chromatographic analysis and other tests suggest that the proteolytic activity of *Lernaea cyprinacea* is due to a single enzyme.

Since literary data have been lacking on similar investigations into the digestive system of copepodes, bovine trypsin was used reference preparation. The molar substrate-splitting quotients established in the BAEE: BAPNA and TAME: BAPNA relations were 43 and 78 for trypsin, and 31 and 21 for the *Lernaea* protease, as assessed by us.

The estimated MW of the *Lernaea* enzyme (about 14 000) was lower than that of the bovine trypsin (23 400); this was a considerable difference between the two enzymes.

The activation energy and the Michaelis constant of the *Lernaea* protease were of the same order as those assessed for bovine trypsin.

As to the other properties of the enzyme, it became markedly activated by certain metal ions (Co^{2+}, Mn^{2+}), while its action was completely depressed by EDTA. Bovine trypsin behaves similarly, even as regards suspension of activity and inhibition.

Examinations with active-group inhibitors have suggested that the enzyme resembles serine protease (DFP-inhibition) and trypsin (TLCK-inhibition) in nature. Its behaviour towards metal ions and its splitting activity on certain synthetic substrates support the trypsin-like nature of the enzyme.

It is remarkable that while the digestive system of other species contains both trypsin and chymotrypsin, that of *Lernaea cyprinacea* contains no chymotrypsin-like protease, as shown by the absence of any indication of BTEE-splitting activity.

It may be concluded that *Lernaea cyprinacea* does possess an independent proteolytic enzyme system, one trypsin-like enzyme component of which had been the subject of this study. Presumably peptidase enzymes are also involved in further steps of the digestion of host-derived proteins.

Summary

Extracts of the copepode *Lernacea* showed proteolytic activity when tested on haemoglobin, casein and gelatine substrates. Assay on BAEE and TAME substrates revealed a marked esterolytic action, while that on BAPNA a less pronounced amidase-like action. No BTEE-splitting action was demonstrable. On chromatographic purification of the extract on Sephadex G-75 column, the enzyme activity was found to be associated with a single fraction, of 14 000 molecular weight. The enzyme activity had its peak at pH 9.0 in TRIS—HCl, phosphate

and borate buffers alike. The Michaelis constant of the Lernaea protease was assessed as 6.8×10^{-6} M, its temperature optimum as 70 °C. Ca^{2+}, Mg^{2+} and Zn^{2+}, as well as PCMBA and fish serum (trypsin inhibitor) had no influence on the enzyme activity, while Co^{2+} and Mn^{2+} increased it, and EDTA, DFP, TLCK, soybean trypsin inhibitor and *Ascaris suum* trypsin inhibitor depressed it to different degrees. The Lernaea protease seems to be a trypsin-like enzyme.

Acknowledgement

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EXPERIMENTAL STUDIES ON EARLY-WEANED LAMBS

I. DEVELOPMENT OF GASTRIC DIGESTION AND METABOLIC CHANGES DURING WEANING

By

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The artificial rearing and early weaning of lambs are important preconditions of large-scale sheep production, but their techniques have not yet been fully established. The improvement of methodical approach presupposes the extension of physiological knowledge on the undeveloped digestive functions of the ovine species. This prompted us to investigate the following aspects of the problem: development of gastric digestion and metabolic changes during weaning; nitrogen and amino acid metabolism in early-weaned lambs; haematological appraisal of protein and amino acid nutrition on the basis of blood parameters; energy- and gas-metabolism in early-weaned lambs.

Experimental observations of the development of gastric digestion in lambs and on metabolic changes related to weaning are reported in this paper. The methodical approach employed was largely based on that used in earlier investigations into the ruminal digestion and metabolism of suckling calves (Szegedi et al., 1974; Juhász et al., 1976).

Materials and methods

Merino lambs 2—3 days old, reared artificially on milk replacer (Phylaxia, Budapest) were kept individually in our animal house.

Before weaning a lamb prestarter had been fed ad libitum. Consumption of milk replacer and dry feed were determined, and the weight gain was followed up. The milk replacer contained 97.85% dry matter, 5.21% ash, 26.23% crude protein and 22.14% crude fat.

The experiments were performed in two series. In *series A*, the ruminal and abomasal digestive functions were studied in 9 lambs, each provided with a ruminal and abomasal fistule, from which samples were withdrawn before and after feeding on days 9, 15, 21, 24, 28 and 34 following upon healing of the surgical wound. The samples were analysed for certain components of ruminal

and abomasal fluid, and the body weight and feed consumption were determined at each sampling.

In *series B*, certain blood parameters were determined to evaluate metabolic functions. Twelve lambs were used in three groups, each consisting of four animals. Blood samples were withdrawn from the jugular vein three hours after feed intake, three times every week between 4 and 38 days of age.

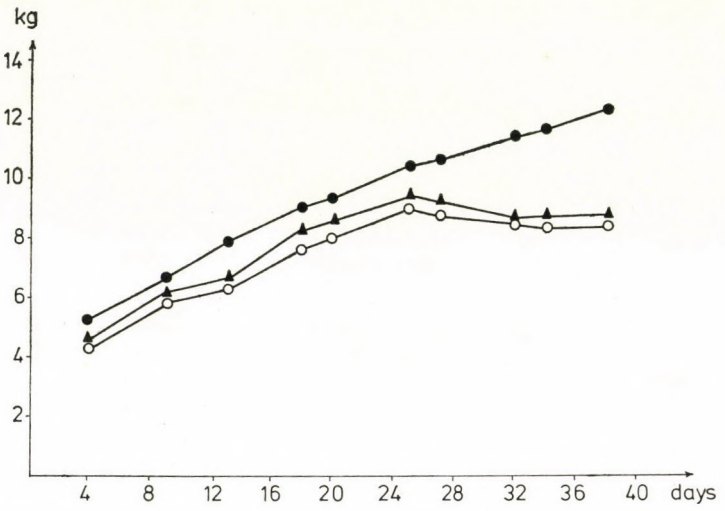

Fig. 1. Weight gain between day 4 and day 38 ●——● Group I; ○——○ Group II; ▲——▲ Group III

The following regimes were employed in the three experimental groups:

Group I: The milk replacer ration was reduced slowly from day 16, and a solid feed was made available from day 13 on.

Group II: The milk replacer ration was reduced from day 24, but more rapidly than in group I, and solid feed was made available from day 17 on.

Group III: The milk replacer ration was reduced gradually from day 13, after supplementation with lysine and methionine, and adjustment to the amino acid level found in ewe's milk. Solid feed was made available from day 10 on (Figs 2 and 3).

The pH of the ruminal and abomasal fluids was determined with a Radiometer PHM 27 apparatus, the total lactic acid level with DISCHE and LÁSZLÓ's (1927) method as modified by us. Abomasal total acidity and chloride content were measured by titrimetry, using a Radiometer PHM 27/ABU 12 apparatus and calomel/glass and calomel/platinum electrodes, respectively.

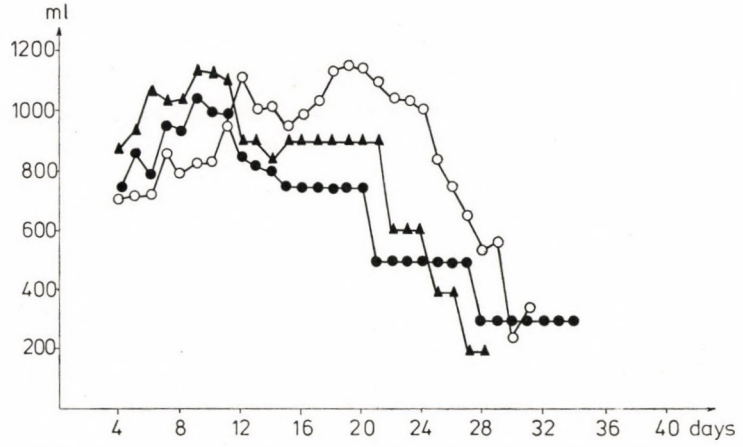

Fig. 2. Milk replacer consumption in groups I—III. ●——● Group I; ○——○ Group II; ▲——▲ Group III

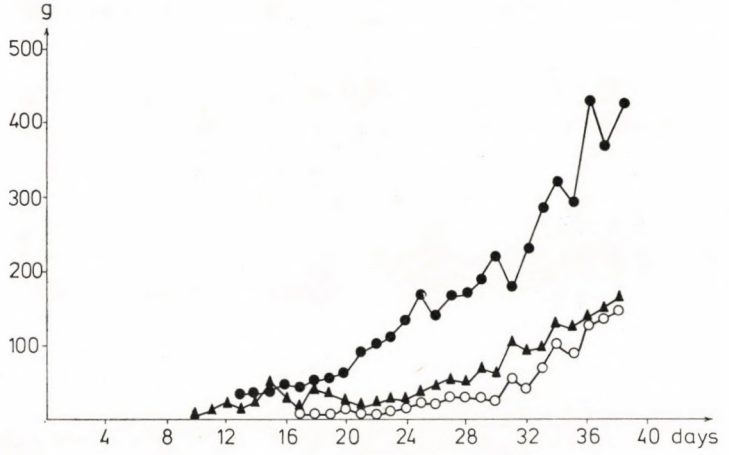

Fig. 3. Dry feed consumption in groups I—III. ●——● Group I; ○——○ Group II; ▲——▲ Group III

The erythrocyte and leucocyte counts of the blood samples were determined in a Buerker counting chamber; haemoglobin was determined by spectrophotometry as proposed by VAN KAMPEN et al. (1961), ammonia by the micro-diffusion method of JUHÁSZ and SZEGEDI (1958), total lactic acid as above, and L-lactic acid by the technique of PARIJS and BARBIER (1964). The blood glucose level was assessed by the orthotoluidine technique (HYVÄRINEN and NIKKIGÄ, 1963), the cholesterol level according to WATSON (1960). Of the inorganic components, inorganic phosphorus was determined by spectro-

photometry by the phospho-molybdic-acid technique, while serum calcium, sodium and potassium were measured by flame photometry (ELERT, 1954).

All test results were plotted graphically and evaluated by biometric methods.

Results

Series A

Samples of abomasal and ruminal contents were taken at different points of time before and after intake of the milk replacer. With 9-day-old lambs sampling and subsequent chemical analysis covered the second or third daily substitute feeding, while in the further experiments they were commenced at the first intake.

Abomasum

At 9 days the abomasal pH rose upon intake of the milk replacer, but subsequently dropped owing to increase of gastric acid production. The same phenomenon occurred on day 15, but from day 20 or 24, the replacer intake no longer raised the pH, which further on persisted about 3.0 (Table II).

Abomasal acidity followed the fluctuations of the pH in each feeding period.

Drop of the pH was always accompanied by a rise of abomasal total acidity (Table III).

The abomasal Cl^- content showed similar fluctuations to total acidity, with significant alterations whenever the acid level changed significantly.

Rumen

The pH of the ruminal fluid changed scarcely between days 9 and 21, and always approximated the pH of the milk replacer. A significant rise, which persisted further on, was first observed on day 24. Standard deviation was nevertheless increasing, for on day 34 there was a drop of pH, followed by its rise again (Table IV).

Ruminal total lactic acid had not changed by persisted at a day 21, but significantly increased thereafter (Table V).

Series B

In all three groups the blood parameters were determined simultaneously with the intake of replacer and solid feed. This system enabled us to correlate the blood parameters with the results of digestive juice analysis (Figs 1, 2 and 3).

Table I

Weight gain and feed concumption of lambs provided with ruminal and abomasal fistules

Age in days	Body weight kg±S	Number of daily feedings	Milk replacer ration (ml)	Dry feed
9	6.35 ± 0.75	5	200	—
15	7.80 ± 0.82	4	200	40
21	9.25 ± 1.05	3	200	90
24	9.95 ± 0.95	2	250	150*
28	10.90 ± 1.22	1	250	220*
34	12.15 ± 1.34	—	—	340*

*Hay *ad libitum*.

Table II

Digestion-related changes of abomasal pH in lambs of different ages

Age in days	Minutes after pail-feeding	pH	s	P *vs.* 0 min
9	0	2.02	±0.47	—
	45	5.01	±0.81	<0.05
	180	2.42	±0.85	>0.05
	225	4.29	±0.23	<0.05
	360	2.80	±0.90	>0.05
15	0	5.05	±0.86	—
	135	2.76	±0.16	<0.05
	225	4.31	±0.13	>0.05
	360	2.78	±0.46	<0.05
21	0	3.72	±0.49	—
	90	4.26	±0.36	>0.05
	210	3.62	±0.52	>0.05
	285	4.39	±0.54	<0.05
24	0	4.37	±0.08	—
	120	3.65	±0.33	>0.05
	300	2.80	±0.44	<0.05
28	0	2.94	±0.44	>0.05
	120	2.91	±0.04	>0.05
	300	2.28	±0.53	
34	0	2.96	±0.44	—
	180	2.73	±0.51	>0.05
	360	3.02	±0.57	>0.05

Table III

Digestion-related changes of abomasal acid content in lambs of different ages

Age in days	Minutes after pail-feeding	Acid content maeq/100 ml	s	P *vs.* preceding sample
9	0	7.35	±0.58	—
	45	1.99	±1.33	<0.05
	180	7.36	±0.47	<0.05
	225	3.20	±0.31	—
	360	6.36	±1.23	<0.05
15	0	4.17	±2.18	—
	135	8.91	±0.90	<0.05
	225	6.86	±0.78	—
	360	10.16	±0.87	<0.05
21	0	5.95	±0.56	—
	90	4.74	±0.54	<0.1
	210	7.66	±1.29	<0.05
24	0	5.24	±0.20	—
	120	7.14	±1.54	<0.05
	240	9.92	±1.29	<0.05
28	0	8.58	±1.12	—
	300	6.75	±1.59	<0.05
34	0	11.15	±2.43	—
	360	10.34	±2.40	<0.05

Table IV

Fluctuations of rumen pH in lambs of different ages

Age in days	maeq/100 ml	s	P *vs.* preceding sample
21	6.65	±0.07	—
24	6.81	±0.04	0.05
28	6.89	±0.15	0.05
34	6.79	±0.25	0.05

Body weights

Inter-group difference in weight gain was significant ($P < 0.05$) between groups I and II, and it was considerable ($P < 0.1$) between groups I and III. In each case, a notable variation of weight gain occurred only after day 25 (Fig. 1).

Table V

Fluctuations of total lactic acid in the rumen content

Age in days	maeq/100 ml	s	P *vs.* preceding sample
21	0.26	±0.03	—
24	0.50	±0.02	0.05
28	0.70	±0.13	0.05
34	0.65	±0.07	0.05

Feed consumption

Group I: Conform to the experimental requirements, the intake of milk replacer rose from 750 to 1050 ml between days 4 and 9; and days 21—27 and 28—34 the ration was reduced to 500 and 300 ml, respectively.

Solid feed intake rose gradually from day 13 on, conform to the demand from 45 to 400 g (Figs 2 and 3).

Group II: Milk replacer consumption rose gradually between days 4 and 12 from 700 to above 1000 ml, and persisted at the latter level until day 24. The replacer ration was then reduced gradually over a period of seven days, until its withdrawal on day 31. Solid feed was made available only from day 17; the daily intake of it did not rise above 100 g until day 34, and scarcely attained 150 g by the end of the experiment (Figs 2 and 3).

Group III: Milk replacer consumption, 1150 ml daily, was higher than in the other groups. Between days 12 and 21 it was reduced to 900 ml, subsequently at intervals of 2—3 days to 600, 400 and 200 ml, until from day 28 on only a solid feed supplemented with amino acids was fed. After withdrawal of the milk replacer, solid feed intake was initially low; it did not rise above 100 g until day 31, although the prestarter was made available *ad libitum* from day 10 (Figs 2 and 3).

Blood parameters

The *haemoglobin* concentration tended to decrease in all three groups, in groups II and III as long as until 22 days, and in these it persisted at a significantly lower level than the baseline until the end of the experiment (Fig. 5).

The *erythrocyte count* tended to decrease in group I, but from day 13 on it rose gradually above the baseline. In groups II and III it dropped temporarily, then rose, but failed to increase over the initial value throughout.

The *leucocyte counts* ($4.4—6.5 \times 10^3/\mu l$) showed no notable alteration during the entire period of study.

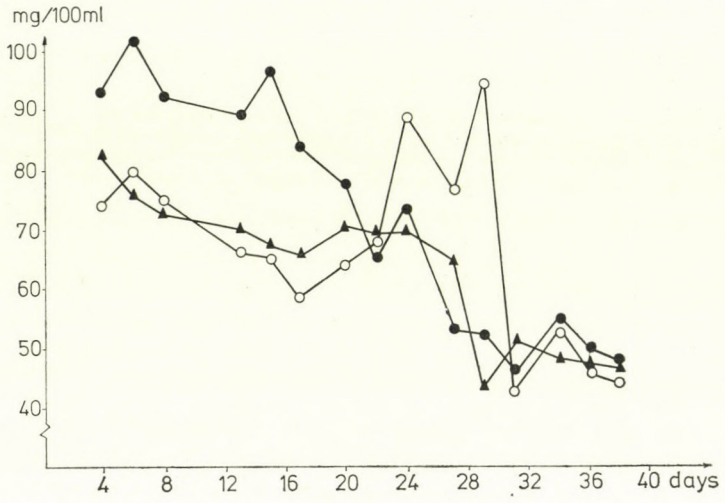

Fig. 4. Fluctuations of blood glucose level in groups I—III. ●——● Group I; ○——○ Group II; ▲——▲ Group III

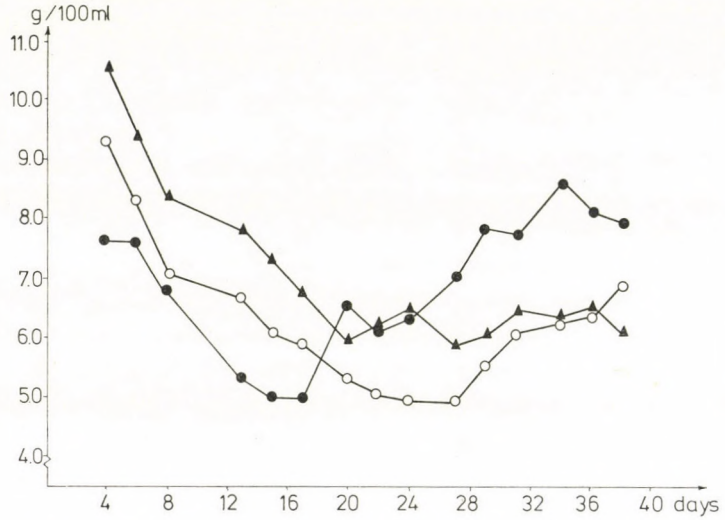

Fig. 5. Fluctuations of haemoglobin concentration in groups I—III. ●——● Group I; ○——○ Group II; ▲——▲ Group III

The *blood ammonia level* (80—250 μg/100 ml) showed no change in group I, while in groups II and III maxima were observed on days 17 and 22, respectively, but neither peak was significant.

The *total lactic acid* concentration (17—35 mg/100 ml) rose significantly in groups I and II until day 15, but subsequently fell until it stabilized around the initial value in all three groups.

Blood *L-lactic acid* content (13—30 mg/100 ml) was somewhat less than total lactic acid, and showed minor concentration changes.

The *blood glucose level* was high in the initial stage of the experiment, and it assumed the low value characteristic of ruminants on days 26, 31 and 29 in groups I, II and III, respectively (Fig. 4).

The lambs of group II showed a considerable rise in blood glucose between days 24 and 29, while those of group III showed a very slight decrease in it until day 24.

The *cholesterol level* fluctuated in the range between 70 and 140 mg/100 ml; its mean values assessed on postnatal days 4 and 31 were 76 and 130 mg/100 ml, respectively.

The serum *sodium* (Na^+), *potassium* (K^+), *calcium* (Ca^{++}) and *inorganic P* levels showed no notable change during the period of study.

Discussion

The artificial rearing of lambs involves two fundamental aspects of digestion physiology: firstly, abomasal digestive activity, which is indispensable for material and energy supply, should be maintained by feeding a milk replacer similar to ewe's milk in composition; secondly, all conditions required for the adequate postnatal development of the forestomachs should be furnished, and a good weaning practice should be employed, in order to make possible the intake of the necessary quantity of solid feed. Both physiological processes are closely associated with the ruminant-specific transformation of the metabolism. The rearing system should be carefully adjusted to these physiological needs. The problems of lamb weaning were studied in great detail by VERESS and KAKUK (1972), who showed in a review (KAKUK and VERESS, 1973) that very varied rearing techniques have been employed, sometimes without consideration to the physiological particularities of the lamb. FREDERICKSON et al. (1971) reported that lambs abruptly weaned when weighing 12.3 kg developed earlier to a finishing weight of 26 kg than those weaned gradually after reaching 14.5 kg body weight. PELLE (1975) believes that despite the higher weaning weight of ram lambs, there is no sex difference in response to weaning.

The development of the ruminant stomachs has been studied by several authors. WANDROP (1960) observed that young ruminants fed on milk developed a small, thin-walled rumen. Experimental feeding of crushed wood (HARRISON et al., 1960) and other materials (TAMATE et al., 1962) showed that the intake of these resulted in increase of ruminal volume and the muscles of rumen wall. WARNER and FLATT (1965) reported that mechanical and chemical stimuli equally had an impact on forestomach development.

Development of the ruminal mucous membrane occurs above all by response to chemical stimuli. Short-chained volatile fatty acids promote the development of the mucosal papillar system. Neomycin or bacitracin depress the function of the bacterial flora, accounting thereby for reduced fatty acid synthesis and consequent smoothness and thinness of the mucosa. SUTTON et al. (1963) observed in calf that development of ruminal absorption is promoted by volatile fatty acids. On a milk diet volatile fatty acid absorption takes place at a very slow rate.

To obviate such risks, in the present experiments the milk replacer was supplied periodically, and solid feed was made available *ad libitum*. The milk replacer ration was risen gradually from 100 ml to 200—250 ml.

Follow-up of the passage of milk replacer by means of the ruminal and abomasal fistules showed that the ingested milk replacer enters through the oesophagus not only into the abomasum, but a notable amount of it appears in the rumen.

Before pail-feeding 9-day-old lambs had a low abomasal pH, ranging between 2.0 and 3.0. After intake of the milk replacer, the pH rose to 4.0—5.0, then tended to decrease again as digestion was progressing. The same change was established after a next 180-min pail-feeding period. The low initial pH may be explained by the fact that in the 9-day-old lamb group the test periods were timed with the second and third daily feedings. Rise of the pH is associated with a reduction of the acid and chloride levels.

In the group of 15-day-old lambs, the first and second feeding periods were followed up. Prior to the first pail-feeding in the morning, the pH was about 5.0 in the resting abomasum. It did not change immediately after intake of the milk replacer, but 135 min later it was found at a low level. The next supply of milk replacer again resulted in a dilution of the abomasal content, followed by changes similar to those found in the 9-day-old group.

On day 24 the acid and chloride diluting effect of the milk replacer was less pronounced; both the total acid and the chloride concentration rose after intake.

From day 28 the abomasal acid concentration persisted at a high level even after intake of the milk replacer. No significant fluctuations took place, suggesting full development of the ruminal digestive function and establishment of a continuous nutrient transport to the abomasum by this time.

The chemical analysis of the *ruminal contents* substantiated the conclusions derived from the observation of the abomasum. The *pH* of the ruminal fluid was constant until day 21, at the same level as the pH of the milk replacer. Only small quantities of solid food were found in the rumen. On day 24 the rumen pH slightly surpassed the initial level; the difference, owing to the low dispersion, was significant, and the lactic acid concentration rose also significantly. Lambs 28—34 days old showed a rumen pH of 6.80—6.90 with

marked fluctuations. A significant increase in the total lactic acid signified that the ruminal digestive function had become established, *i.e.* the fermentation processes had commenced.

It may be concluded that the development of ruminal function took 24 days under the given conditions (Table IV).

Under natural conditions, the suckling lambs take up their daily ration of ewe's milk by suckling the dam on 20—24oc casions. GAÁL and CZAKÓ (1973) observed in behavioral studies on artificially reared lambs 15 sucklings a day at 20 days of age. STEPHENS and BALDINI (1971) reported that 1—3 weeks old lambs supplied themselves from the self-operated feeder on five occasions, while 4—5 weeks old ones on four occasions within a 6-hour period. During a single suckling lambs take in a little amount of milk, with which the abomasum can easily cope. In the rumen only solid food is accumulated; its bulk and its degradation products stimulate ruminal development. Periodic large supplies of milk replacer from the pail fill the developing rumen as well. After filling of the abomasum, the excess fluid is accumulated in the rumen, and presumably retards the onset of ruminal digestion. Formerly, this phenomenon was regarded as pathological, but apparently, it does not give rise to indigestion under the pail-feeding system. Analysis of our experimental findings in the light of behavioral observations has led to the conclusion that a continuous supply from a self-operated feeder, which the lambs use *ad libit.* might probably be more advantageous in artificial lamb rearing than periodic feeding.

The establishment of the ruminal digestive activity accounts for an alteration of the inner metabolism resulting in the development of a characteristic intermediary metabolism which differs in many respects from that operative in non-ruminants. Inadequate transformation, on the other hand, accounts for an altered growth rate and concentration changes of certain blood constituents, and may also give rise to indigestion and/or metabolic disorders.

Under the given conditions of experiment, the milk replacer diet was associated with a depression of haemopoiesis during the first two postnatal weeks as shown by a gradual, but ultimately significant, decrease in the haemoglobin concentration. Increasing intake of solid food and onset of ruminal digestion enhanced the synthesis of haemoglobin until by day 38 it rose above the initial level (Fig. 5).

According to HORVÁTH and NACSEV (1972), newborn calves develop a physiological anaemia characterized by a considerable decrease in the haemoglobin concentration for 4—6 weeks, and a subsequent tendency to normalize owing to increasing intake of solid feed. BAUMGARTNER et al. (1972) observed a distinct anaemic state (18 ppm Fe/ml) in calves fattened on milk replacer. Physiological anaemia was developed by our lambs as well, during the milk substitute regime and it normalized slowly as solid feed intake increased. In

the other two groups, where transition to the solid feed regime was delayed, the haemoglobin concentration and the erythrocyte count had remained at low levels by the end of the experiment.

The present studies have disclosed an interaction between the haemoglobin concentration and erythrocyte count on the one hand and the weaning-induced changes of inner metabolism on the other. This interaction is naturally influenced by solid food intake and ruminal digestion. Postnatal physiological anaemia, undesirable in artificial lamb rearing, can be prevented by an appropriate treatment with iron preparations. Thus, complications after weaning can be avoided and the subsequent growth will be speeded up.

Among the postnatal metabolic alterations characteristic of ruminants, those of the carbohydrate metabolism are particularly pronounced in the weaning period.

CHYLA et al. (1970) assessed the blood glucose level of lambs as 71.7, 85.9, 45.8 and 51.4 mg/100 ml at 10, 20, 30—35 and 40—45 days of age respectively.

Furthermore, LUPIEN et al. (1962) established that the usual drop of blood glucose also took place after removal of the forestomachs. NICHOLAI and STEWART (1965) introduced the feed directly into the abomasum, but this did not prevent the blood glucose drop. WALKER (1959) suggested that the lactase enzyme activity was responsible for the low blood glucose. In our studies the lambs had a reduced glucose level (about 50 mg/100 ml) around the time of onset of ruminal digestion. In groups II and III, where the solid feed supply was delayed the blood glucose drop took place later, on days 29—31. In group II the reduction of the milk replacer ration even accounted for a hyperglycaemic episode of several days. A temporary slight hyperglycaemia occurred in group III, too (Fig. 4).

The blood levels of total lactic acid and L-lactic acid showed no notable change during the experimental period; any fluctuation observed was within the physiological range. The proportion of the lactic acid components nevertheless changed: the L-lactic acid concentration fell, while the total lactic acid rose, owing either to the increasing ruminal absorption of lactic acid, or to a better utilization of L-lactic acid.

The blood cholesterol level tended to rise during the experimental period, significantly so in groups II and III around day 31. Rise of the cholesterol level involves lipid mobilization. The material and energy supplies are inadequate at this stage of weaning. The associated of rising cholesterol and elevated blood glucose levels has suggested a predominance of mobilizing metabolic processes.

Summary

Abomasal function, development of ruminal digestion, and blood parameters conclusive of metabolic changes were examined from 4 to 38 days of age in early-weaned, artificially reared Hungarian Merino lambs. The effects of weaning on digestion and metabolism were studied under three different regimes of periodic pail-feeding.

The periodic supplies of milk replacer accounted for filling not only of the abomasum, but partly also of the rumen, for their quantity was greater than the abomasal volume. Intake of milk replacer was followed by a marked alteration of pH, and of acid and chloride levels in the abomasum. This was probabily responsible for the retardation of the development of ruminal microflora and microfauna, and of digestive fermentation. During weaning, the developing ruminal digestive mechanism serves as auxiliary to abomasal digestion; the latter should be supported by adequate nutrient supplies until the digestive mechanisms characteristic of the adult sheep become established in both forestomachs.

Adequate reduction of the milk replacer ration promoted the intake of solid feed; overdosing or abrupt withdrawal of the former caused metabolic alterations. Ruminal digestion may become disturbed, or even pathologically altered, during weaning and may give rise to disorders of protein, carbohydrate, fat and mineral metabolism. The postnatal metabolic transformation, which is characteristic of ruminants, took an uncomplicated course if the weaning-related diet change caused neither nutrient nor energy deficiency. In the reverse case, the physiologically low blood glucose level characteristic of the adult ruminant became established with a delay, and the blood cholesterol level rose owing to compensation of energy deficiency by mobilization of the fat reserves.

During the first two weeks, the lambs developed anaemia which, however, ceased gradually at the onset of normal ruminal digestion. Under inadequate conditions of weaning the anaemia took a prolonged and more severe course, accounting for developmental retardation. Prevention of lamb anaemia by iron preparations is recommended in the interest of even development.

In artificial rearing conditions a continuous supply of milk replacer from a self-operated feeding device seems to be closer to the physiological conditions than periodic feeding from the pail.

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EXPERIMENTAL STUDIES ON EARLY-WEANED LAMBS

II. NITROGEN AND AMINO ACID METABOLISM

By

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Investigations into the physiological aspects of digestion and metabolism in artificially reared, early-weaned lambs were reported in the first part of this publication series (SZEGEDI et al., 1980).

Further studies on the nitrogen and amino-acid metabolism of lambs during early weaning and subsequent artificial rearing are described in this paper.

Until the completion of forestomach development, the suckling lamb can well be regarded as a monogastric animal. For this reason, coordination of the digestive and metabolic functions requires particular attention during the critical early period.

Early weaning of lambs generally means that lambs are weaned and change to a milk-replacer diet at 1—2 days of age. In a literary review on the early lamb weaning practice, KAKUK and VERESS (1973) pointed out the great contradictions in pertinent recommendations. In the United Kingdom abrupt weaning has been preferred without a preparatory period, while in other countries either the body weight, or the quantity of ingested solid feed has been observed as a criterion. Generally three weeks have been regarded as the earliest age for withdrawal of the milk supply. GAÁL (1968, 1969) reported a very good growth performance under field conditions with an appropriate strategy of early weaning and solid feed regime. ØRSKOV et al. (1971) established that the feed consumption of the growing lamb depends not only on ruminal fermentation, but also on the dietary protein level. In another study, ØRSKOV et al. (1973) found 33 days to be the optimum weaning age, with regard to the effects of weaning time on feed utilization.

Information has been scanty on the amino-acid composition of milk replacer and on the nitrogen and amino-acid metabolism of lambs during the first postnatal weeks. The data presented by HOGAN (1970) on ovine amino-acid requirements at different ages cover all age groups except the youngest lambs. KOLENKO et al. (1973) reported that supplementation of the lamb diet with DL-methionine and L-lysine enhanced microbial fermentation and protein synthesis in the rumen. On examining different dilutions of milk replacer BAUER et al. (1971) found that lambs given a replacer containing 26% crude protein and 18% fat at 200 g/litre dilution gained, on the average, 199 g weight daily. They suggested a weaning weight of 12 kg, and a daily solids intake of 200—250 g as optimum conditions for milk withdrawal; the lambs satisfying these criteria are usually 35—40 days old. LEMAY (1973) recommended 25% crude protein and 30% fat levels for the ewe's milk replacer, and 4—6 weeks as optimum age for milk withdrawal.

NIMRICK et al. (1970) established the sequence of limiting dietary amino acids for the growing lamb as methionine, lysine and threonine. KOSAROV et al. (1975) proposed that the

lysine content of the milk replacer should be adjusted to the level naturally occurring in ewe's milk. BETRAM et al. (1974) observed that orally-administered amino acids become degraded in the rumen, but steraoyl-methionine breaks down only in the abomasum. HATFIELD (1970), too, advocated the importance of adequate amino-acid supplementation on the grounds that the type and quantitative relations of the absorption of dietary amino acids have a major impact on performance. In studies of lamb growth from two days of age, SEARIE and GRAHAM (1972) found on indication of lipid incorporation during the period of rumen development.

JORDAN and TICHICH (1973) recommended for early weaned lambs a high-energy fodder diet which, if formulated adequately, may render the hay ration unnecessary. According to TARAKANOV (1974), methionine and lysine enhance cellulose digestion in ruminants, but excessive methionine supplies developed inhibitory action.

To reconcile the conflicting views expressed on lamb rearing by different authors, we experimented with ram lambs under artificial rearing conditions. Feed consumption and weight gain were determined, and the nitrogen and amino acid metabolism was followed up with special regard to lysine and methionine retention, in order to assess their value as growth parameters.

Material and methods

Sixteen ram lambs divided in three experimental groups were used. Each lamb was lactated by the ewe until two days of age and had, accordingly, taken up the available colostral antibodies before change to a milk replacer (PHYLAXIA, Budapest) after early weaning. Each experimental group was put to a different diet regime. The milk replacer was supplied at 250 g/litre dilution, and a pelleted fodder prestarter, formulated by us, was additionally fed. The milk replacer was diluted with regard to its dry matter content (20—22%). The replacer was pail-fed on five occasions daily, at 7 and 10 a.m., and 1, 4 and 7 p.m. Drinking water was made available *ad libitum* from the beginning of the feeding experiment. The composition of the prestarter, and the constituents of the milk substitute and of an average batch of ewe's milk are shown in Table 1.

Table I

Timing of the test periods

Period No.	Group I	Group II	Group III
	age in days		
1	5—10	4— 9	4— 9
2	16—21	12—17	12—17
3	23—28	20—25	20—25
4	30—35	28—32	28—32
5	38—42	36—40	36—40

Laboratory analysis revealed a considerable difference between the constituents of the milk replacer used, and ewe's milk. While the replacer contained, on the average, 24% crude protein, the freeze-dried ewe's milk, with a dry matter content of 90%, contained 31%, as well as 2 and 0.7 g more lysine and methionine than the substitute. The available lysine content was also higher, although not significantly, in ewe's milk (93.9%) than in the replacer (89.8%).

The experiment lasted until 40 or 42 days of age and included five test periods, each five days long, during which the quantity of feed intake, and urine and faeces discharge were assessed daily to follow up nitrogen and amino acid metabolism. The timing of the test periods is tabulated in Table I.

Ingested and excreted nitrogens was measured on each test day to assess N-retention. Methionine and lysine retention were calculated from the difference between the amounts ingested and eliminated with the faeces. The five-day test periods were always separated by transitory rest periods, during which no measurement was performed.

Nitrogen was determined with the Kejdahl method, while amino acids in a Bio-Cal 200 automatic analyser.

Table II

The use of milk replacer and prestarter

Age in days		Milk replacer rations ml/lamb	Consumption of	
			milk replacer	prestarter
			ml	g
Group I.	2—12	ad lib.	1048	—
	13—15	850	850	—
	16—20	750	750	43
	21—27	500	500	101
	28—34	300	300	229
Group II.	1—24	ad lib.	990	—
	25—27	900	900	13
	28—29	600	600	32
	30—32	350	350	35
Group III.	2—10	ad lib.	1019	—
	12—21	900	893	28
	22—25	600	600	22
	26—27	400	400	40
	28—29	200	200	49

Treatment

Group I: Four ram lambs were kept in metabolic cages which made possible the precise quantitative determination of feed consumption, and urine and faeces discharge. The schedule of milk replacer supplies and the data of milk replacer and prestarter consumption are in Table II.

The fodder prestarter was made available *ad libitum* from 13 days of age.

Group II: Six two-day-old ram lambs were transferred to metabolic cages as above. The schedule of milk replacer supplies and the data of milk replacer and prestarter consumption are shown in Table II.

In this series the lamb prestarter was made available *ad libitum* from 19 days of age.

Group III: Six ram lambs were kept in metabolic cages from two days of age as in group II. The Phylaxia milk replacer was supplied in the same

Table III

Composition of the lamb prestarter

Components	%
Barley	32.0
Maize	25.0
Soybean extract	15.0
Skim milk powder	15.0
Alfalfa meal, medium quality	6.0
Powdered fat, "Favorit 50"	4.0
Salt	1.0
Lime	1.0
AP 18 (Phylaxia)	0.5
Standard premix no. XIX (Phylaxia)	0.5

Percentual constituents of PHYLAXIA milk replacer, freeze-dried ewe's milk and lamb prestarter

Constituents	Milk replacer lamb formula	Freeze-dried ewe's milk	Prestarter
Original dry matter	—	21.37	—
Dry matter	92.2	90.16	90.35
Crude protein	24.1	31.33	18.88
Crude fat	29.1	21.37	3.88
Crude ash	5.6	8.97	5.83
Lysine	2.8	4.86	0.78
Methionine	0.7	1.45	0.57
available lysine, rel. %	89.8	93.93	90.50

dilution, but it was supplemented with L-lysine (3.5 g/1000 ml) and DL-methionine (1.2 g/1000 ml), to increase the amino-acid level to that present in ewe's milk. Thus the replacer fed to the lambs of group III represented a modified formula relative to the plain preparation used in Groups I and II.

The data of milk replacer rations and uptake, and of prestarter consumption can see in Table II.

The prestarter diet was made available *ad libitum* from five days of age.

The composition of the lamb prestarter (PHYLAXIA) you can see in Table III.

Results

The live weight gains established over the consecutive test periods are shown in Fig. 1. The lambs of group I showed an almost linear increase of body weight. In group II, the live weight increased evently until 28 days of age, owing to even intake of the milk replacer. The group II lambs were used to test response to a relatively rapid reduction of the milk replacer ration after an *ad libitum* supply there of until 24 days of age. The animals responded to

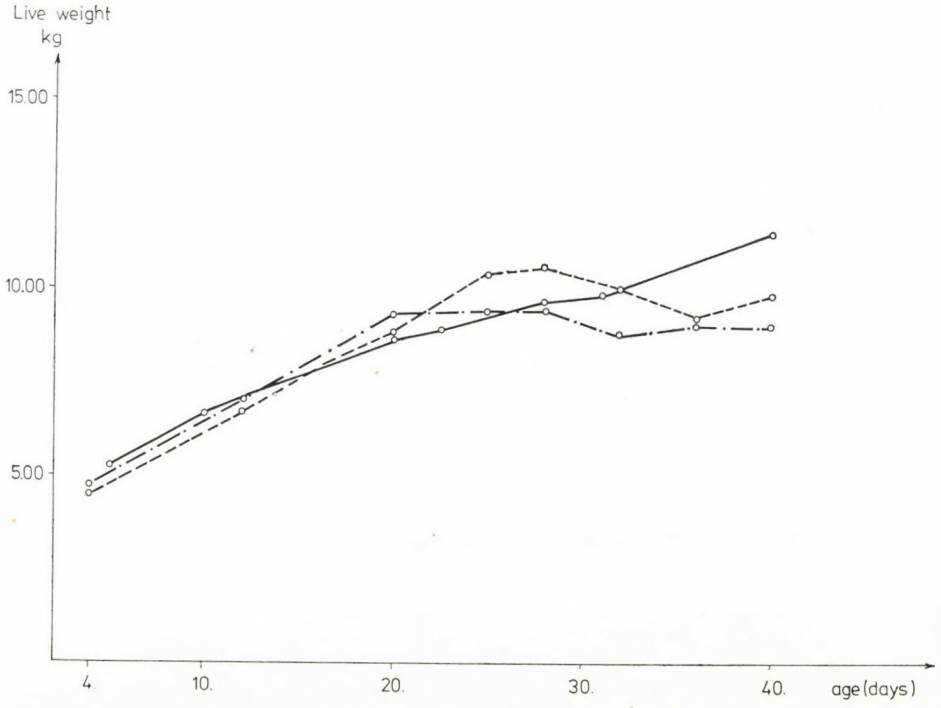

Fig. 1. Changes in live weight during the experimental period. —— Group I; — — — Group II; —.—.— Group III

the rapid withdrawal by retardation of weight gain between 28 and 36 days of age, and did not take in notable amounts of the solid feed until the fifth test period, by which the replacer was completely withdrawn. At this stage, the average daily prestarter consumption rose to 120 g, and accounted for a daily weight gain increment of 37 g. In group III, the lambs ceased to gain

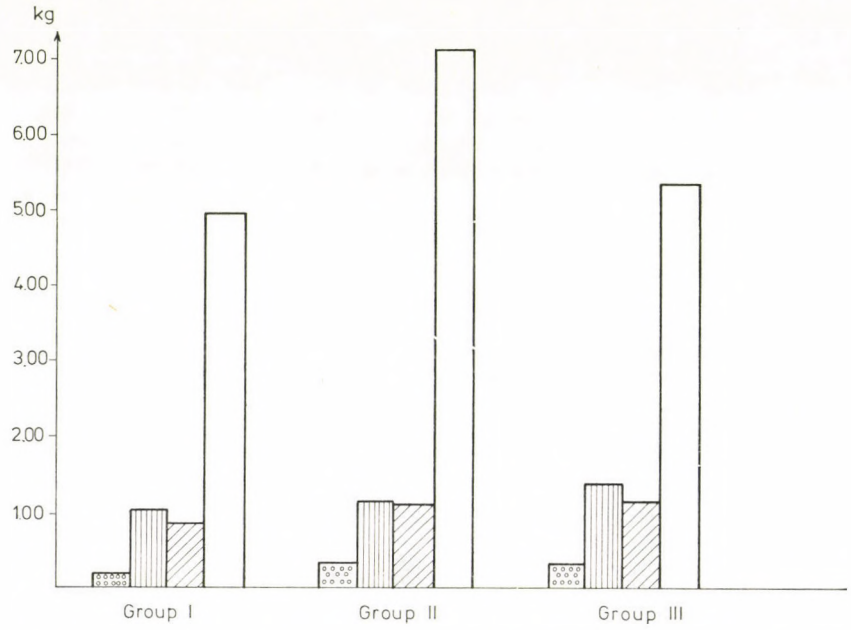

Fig. 2. Quantity of milk replacer utilized for one kg live weight gain. ▨ milk replacer utilized for 1 kg live weight gain; ▭ total milk replacer consumption until weaning; ▭ digestible protein utilized for 1 kg live weight gain; ▭ starch equivalent utilized for 1 kg live weight gain

weight from day 20 — owing to a drastic reduction of the milk replacer ration — and began to thrive again only from day 36.

The average amount of milk replacer utilized for 1 kg live weight gain was 5.0—5.4 kg in groups I and III, indicating a medium utilization. In group II, the corresponding ratio was much higher, 7.2 kg, for the first three test periods wheile replacer had been available *ad libitum*.

The quantities of milk replacer utilized for 1 kg live weight gain in the different experimental groups, and the corresponding starch equivalent and digestible protein values are shown in Fig. 2.

Fluctuation of the daily weight gain and of nitrogen, lysine and methionine retention of the group I lambs are shown in Fig. 3. The daily nitrogen intake decreased from 9.8 to 7.7 g during the first three test periods, then it rose abruptly to 13 g on the average, and remained as high over the fourth and

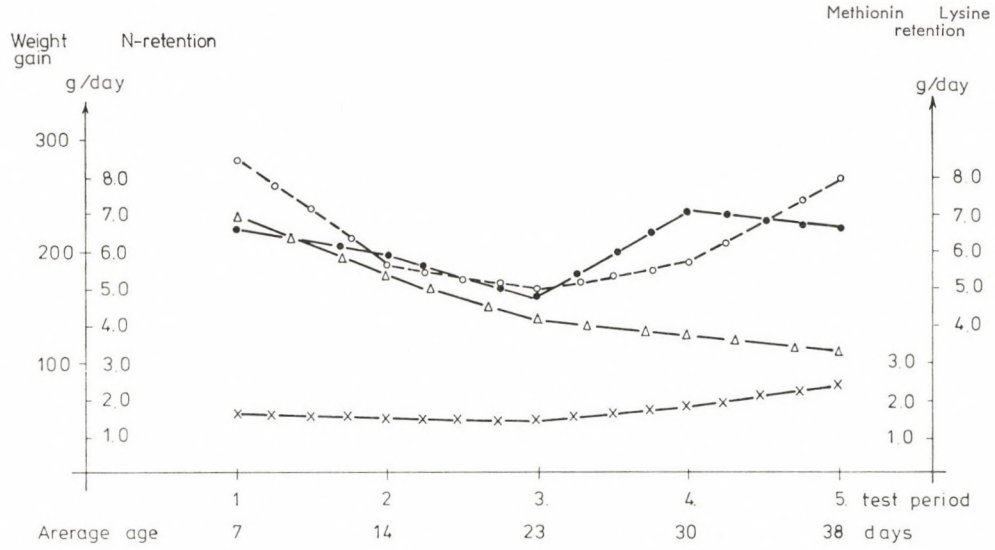

Fig. 3. Retention of nitrogen, lysine and methionine, in Group I. ○— — —○ weight gain; ●——● N-retention; △——△ lysine retention; ×——× methionin retention

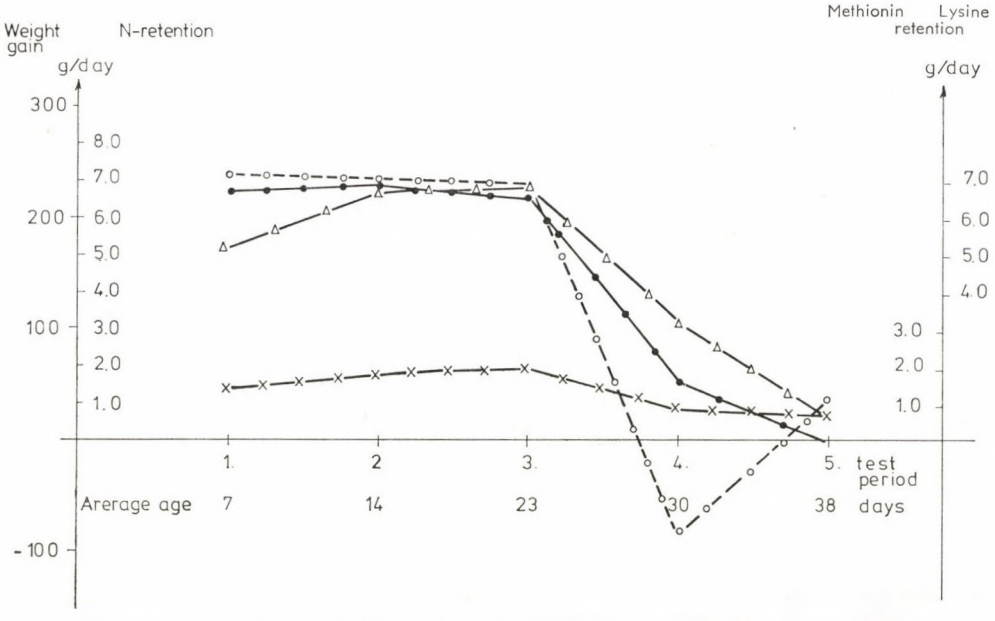

Fig. 4. Retention of nitrogen, lysine and methionin in Group II. ○— — —○ weight gain; ●——● N-retention; △——△ lysine retention; ×——× methionin retention

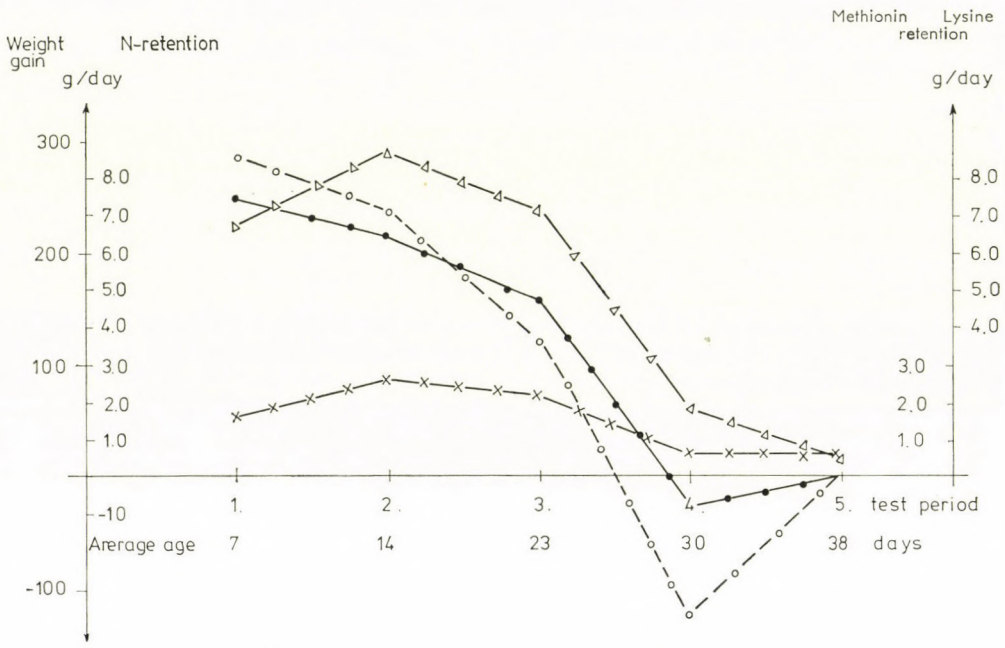

Fig. 5. Retention of nitrogen, lysine and methionin in Group III. ○——○ weight gain; ●——● N-retention; △——△ lysine retention; ×——× methionin retention

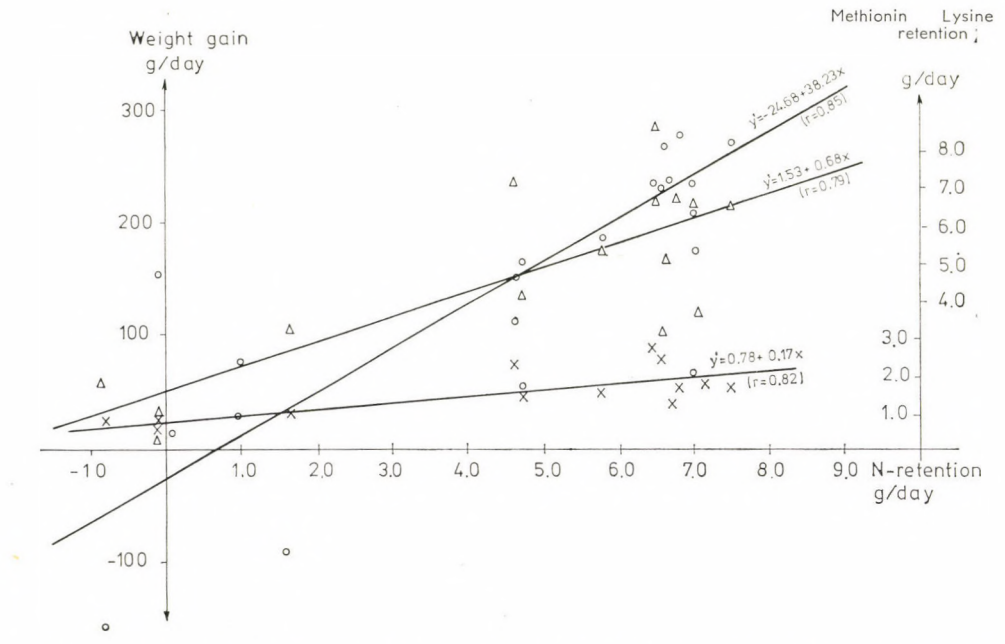

Fig. 6. Correlation of nitrogen retention with weight gain, and with lysine and methionine retention. ○——○ weight gain; △——△ lysine retention; ×——× methionine retention

fifth periods, *i.e.* until 40 days of age. Nitrogen retention decreased from 6.8 ± 1.06 g in the first period to 4.7 ± 1.54 in the third one, then it rose to 6.6 ± 1.18 and 7.1 ± 2.82 in the fourth and fifth periods, respectively. Methionine retention showed no notable change during the first four test periods (1.5 ± 0.33 — 1.8 ± 0.54 g), and rose to 2.5 ± 0.45 only during the fifth period. Lysine retention fell gradually from an initial 6.9 ± 0.68 g in the first period to a daily average of 3.3 ± 0.61 g are in fifth period.

Fig. 4 shows the fluctuations of weight gain, as well as nitrogen, lysine and methionine retention in group II. During the first three test periods the daily nitrogen intake was 9—11 g, while nitrogen retention ranged from 6.5 ± ± 0.91 to 6.9 ± 0.86 g. Owing to the abrupt withdrawal of the milk replacer, the daily nitrogen intake fell to 5.19 and 2.64 g in test periods 4 and 5, respectively, and nitrogen retention was correspondingly reduced to 1.63 ± 0.55 and —0.03 ± 1.69 g. Methionine retention tended to increase slightly from 1.3 ± 0.33 to 2.0 ± 0.26 g during the first three test periods, but subsequently tended to decrease by analogy of the nitrogen retention. Lysine retention showed the same tendency: it ranged between 5.1 ± 32 and 6.9 ± 0.66 g during the first three test periods, and fell to 0.6 ± 0.08 g in the fifth period.

The weight gain and the nitrogen, methionine and lysine retention data assessed in group III are shown in Fig. 5. Amino acid supplementation of the milk replacer failed to improve nitrogen retention. The initial average daily nitrogen intake of 10 g decreased gradually to 2.09 g by the fourth test period, owing to reluctance of the lambs to take in solid feed, and did not increase again until the fifth test period. Simultaneously with the reduction of nitrogen intake, the nitrogen retention tended to decrease gradually from an initial 7.49 ± 0.67 g to —0.8 ± 0.68 g in the fourth test period, during which feed consumption fell below the normal requirement; normalization followed only during the fifth period. Methionine and lysine supplementation had little impact on the retention of either amino acid. Methionine retention was assessed as 1.6 ± 0.24, 2.6 ± 0.39 and 2.2 ± 0.89 g, respectively, in the first three test periods, but it did not rise above 0.7 ± 0.29 and 0.8 ± 0.10 in the last two ones. Lysine retention changed similarly during the five test periods, showing values of 6.6 ± 0.84, 8.9 ± 0.86, 7.1 ± 1.87, 1.7 ± 0.88 and 0.5 ± ± 0.39 g, respectively.

Discussion

Among the lambs reared on milk replacer with or without amino-acid supplement for different times, those of group I showed the most even growth rate and correspondingly even values of nitrogen, lysine and methionine retention, signifying that change from liquid to solid feed did not involve a notable growth arrest under the regime employed. Gradual withdrawal of the milk

replacer ration between 12 and 34 days of age did not interfere with the development and functional activation of the forestomachs, so that the lambs were able to ingest, and digest, the necessary quantities of solid prestarter.

The lambs of group II, although they had developed evenly until 24 days of age owing to the milk replacer available *ad libitum*, showed on abrupt withdrawal of the latter a considerable reduction of the initially even weight gain, and of nitrogen, lysine and methionine retention as well. At 4—5 weeks of age, amino acid retention was very low, ranging from one third to one half of that found in group I. Abrupt withdrawal of the milk replacer temporarily arrested the development of ruminal digestive function at a stage incapable of coping with the utilization of solid feed as material and energy source.

Similarly, unfavourable results were obtained in group III, in which the milk replacer had been supplemented with amino acids to render it equivalent to ewe's milk in respect of lysine and methionine content. Abrupt withdrawal of the milk replacer interfered with digestive adaptation to weaning also under this regime. It follows that the completion of forestomach development, which is an indispensable prerequisite of undisturbed weaning, cannot be accelerated with amino acid supplementation alone.

The fate of ingested dietary proteins and amino acids was followed up in the course of the experimental study, in order to extend the scanty knowledge of their metabolic pathways in sucking lambs. Correlations were therefore pursued between nitrogen retention and daily weight gain as well as methionine and lysine retention.

The relationships found (Fig. 6) can be characterized by the following equations of regression:

$$y = -24.68 + 38.23\times \qquad n = 15 \qquad r = 0.85$$

for nitrogen retention and weight gain,

$$y = 0.78 + 0.17\times \qquad n = 15 \qquad r = 0.82$$

for nitrogen retention and methionine retention, and

$$y = 1.53 + 0.68\times \qquad n = 15 \qquad r = 0.79$$

for nitrogen and lysine retention.

The correlation coefficients and the regression equations unequivocally indicate a close correlation between the tested parameters. In all three cases the correlation was significant at the $P = 0.001$ level. The moderately close relationship between nitrogen retention and weight gain indicates that the

feeding regime satisfied the dietary needs of the animals, for the correlation would have been less close if the animals had displayed either fat or water incorporation in consequence of the diet's low nutritional value.

The experimental observations have shown that the growth disturbing effects of weaning stress can be minimized only by slow gradual withdrawal of the ruminal microflora and microfauna collaborating in solids digestion. The milk replacer offered by the Phylaxia Veterinary Biologicals and Feedstuffs Co. Ltd. proved to be suitable for artificial lamb rearing, for its application made possible an uncomplicated weaning at 32 days of age, after gradual reduction of the replacer ration.

The experiments have also disclosed that if a lamb prestarter containing about 18% digestible protein is offered in addition to the milk replacer, the daily weight gain of the lambs can be increased to 200 g under adequate environmental conditions, during both artificial rearing and weaning.

Summary

Lambs weaned at two days of age after intake of the colostrum were transferred to single metabolic cages, where their nitrogen, methionine and lysine metabolism was followed up during artificial rearing from 2 to 42 days of age. Three groups were formed to test the effects of different milk replacer regimes and weaning ages. The nitrogen, methionine and lysine metabolism was studied over five test periods, each five days long within the 38-day period of study. The correlation of nitrogen retention with weight gain ($r = 0{,}85$), as well as methionine ($r = 0.82$) and lysine retention ($r = 0.79$) was significant at the $P = 0.001$ level.

It is concluded that the growth-disturbing effects of early lamb weaning can be minimized by a cautions gradual withdrawal of the milk or replacer (from 12 to 32 days of age), which makes possible the full development of the ruminal microflora and microfauna indispensable for solid feed digestion.

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CHANGES OF PLASMA PROGESTERONE AND TESTOSTERONE LEVEL IN CHICKEN *(GALLUS DOMESTICUS)* FROM THE FIRST DAY OF LIFE TO THE BEGINNING OF OVIPOSITION

By

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Results of recent of investigations point to the fact that in mammals there is a considerable production of sexual steroids, partly of adrenal origin, even before sexual maturation *i.e.* before puberty.

Maturation is accompanied by changes in sensitivity of receptors in the hypothalamo — hypophyseo — gonad axis resulting in alteration of the quantities of produced hormones (Eldridge et al., 1974; McPherson et al., 1974). The estrogen level has been found to be very high in immature female rats in the first three weeks of life, plasma progesterone concentration at the same time is significantly lower than in mature animals. In the period of prepuberty a continuous rise in progesterone and a low estrogen level were found (Döhler and Wuttke, 1974, 1975; Ojeda et al., 1975). It may be assumed that estrogens are of vital importance in the maturation of the hypothalamo-hypophyseal system in rats (Steele and Weisz, 1974; Ojeda et al., 1975). Estrogens in birds play a primary role in the morphological and functional "preparation" of the oviduct and in the transformation of the calcium and phosphorus metabolism during maturation (Brant and Nalbandov, 1956; Palmiter and Wreen, 1971; Senior, 1974; Sturkie, 1976). In the regulation of the ovulatory cycle presumably progesterone is the most important hormone (Furr et al., 1973; Laquë et al. 1975). We have shown (Péczely et al., 1979) that amidst sexual steroids only progesterone displays a preovulatory surge, which is accompanied by a very low testosterone level. The asynchronous variation of plasma progesterone and testosterone, suggest the distinct role of these two steroids in the maturation of the ovum and in the egg formation.

The present paper deals with concentration changes of the sexual steroids, possessing presumably different targets, during the ontogenesis and confronts the results obtained here with those observed by other workers throughout the ovulatory cycle of the mature, laying hen.

Materials and methods

TR-90 hybrid female chickens, one day, 3, 15, 21 and 22 weeks of age were used. They were kept in 12L—12D photoperiod and fed chicken or laying-hen feed and water *ad libitum*. Blood form the one-day-old and three-weeks-old groups was obtained by decapitating groups of birds at 4-hr intervals

through 48-hr. From older hens about 3 ml of blood was drawn every four hours through 48-hr by means of chronic cannualas inserted into the *v. brachialis*. Corpusculate elements were reinjected every 8 hr after washing them with 0.85% saline. Blood samples collected in heparinized tubes were centrifuged at 3000 rpm, plasma was stored at −20 °C. Since the development of follicles occurred in the 21st week, this group was separated into "adults" and "immatures" according to the phase of the follicles. Pullets began to lay eggs in the

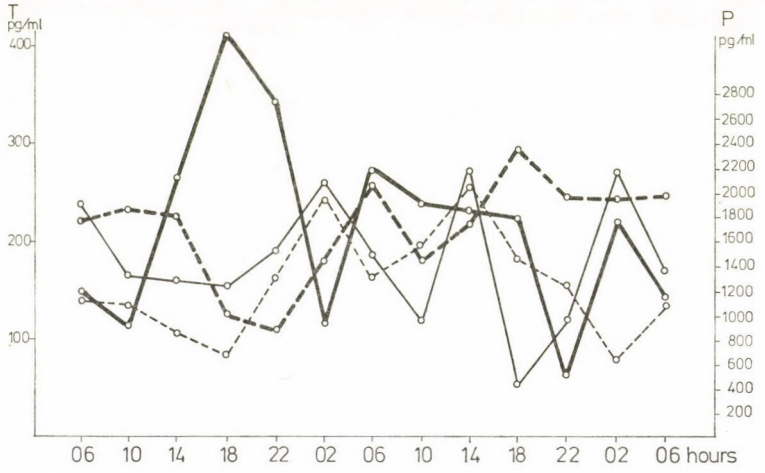

Fig. 1. Fluctuation of plasma progesterone and testosterone levels in chickens no. 1 and 2. aged 15 weeks. ▬▬ testosterone ▬ ▬ ▬ progesterone;

22nd week. Plasma progesterone and testosterone were measured without preliminary separation or purification by RIA according to ABRAHAM et al. (1971) and JALLAGEAS (1975), respectively.

Reliability data and specification of antisera have been published elsewhere (PÉCZELY et al., 1979). Results are demonstrated as individual curves to illustrate daily hormone production qualitatively. Average hormone production and standard deviation (±SD) through 48 hr has also been calculated.

Analaysis of groups of chickens decapitated at 4-hr intervals proved that no diurnal rhythm of progesterone exists from one day to 3 weeks of age.

In the age group from 15 weeks to 21 weeks there appear one or two irregular peaks of progesterone. Comparison of two 24-hr samping periods could not prove any synchronizing factor in the appearance of progesterone peaks neither in nor between birds. While in some birds very low or slightly fluctuating basic levels (262 ± 174 pg/ml) developed during maturation (21 weeks, "adult"), others exhibited irregular peaks (455 ± 234 pg/ml) characteristic of immature birds. Typical preovulatory progesterone surges (1239 ±

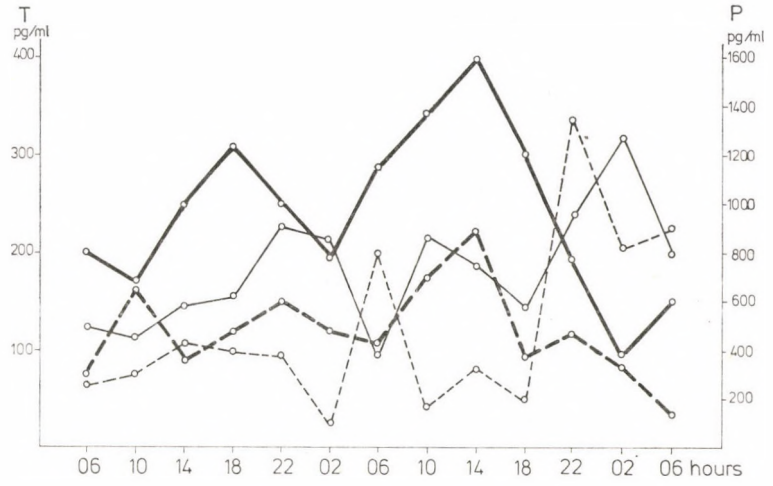

Fig. 2. Fluctuations of plasma progesterone and testosterone levels in immature, 21 weeks old chickens. no. 1 and 2. ▬▬ testosterone; ▬ ▬ ▬ progesterone

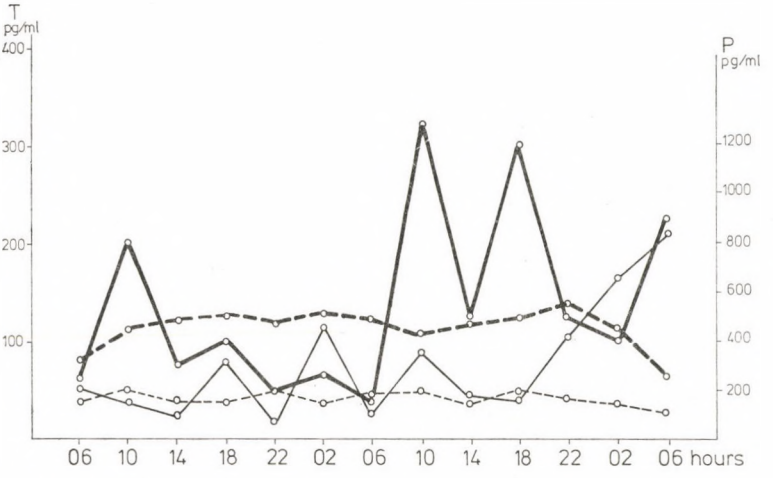

Fig. 3. Fluctuations of plasma progesterone and testosterone levels in mature, 21 weeks old pullets. no. 1 and 2. ▬▬ testosterone; ▬ ▬ ▬ progesterone

$\pm$ 467 pg/ml), starting from a higher basic level, could be detected in 22 weeks old laying pullets.

Plasma testosterone concentration does not reveal significant fluctuation during ontogenesis (Table I).

Mean testosterone concentration of one-day-old and 3-weeks old chicks decapitated at 4-hr intervals shows some irregular variation, but no diurnal

Table I

Changes in plasma progesterone and testosterone levels from the first day of life to the onset of oviposition

Age	Progesterone (pg/ml)	Testosterone (pg/ml)
day old	479 ± 317 (83)	103 ± 35 (83)
3 weeks	873 ± 493 (112)	164 ± 65 (112)
15 weeks	1302 ± 472 (10)	142 ± 79 (10)
21 weeks immature	570 ± 366 (6)	184 ± 86 (6)
21 weeks mature	262 ± 174 (4)	124 ± 77 (4)
22 weeks	1239 ± 467 (4)	116 ± 82 (4)

rhythm. One or two irregular testosterone peaks may be observed daily in birds aged 15 and 21 weeks. These become more regular, appearing at every 8—12-hr in 21 weeks old adult pullets. Occasionally one can observe testosterone peaks 10—12-hr before oviposition in laying pullets aged 22 weeks.

Discussion

Variations in plasma concentration of estrogens during ontogenesis have been studied by several authors. According to SCHRÖCKNADEL et al. (1973), WINELAND and WENTWORTH (1975) estrogen concentration of "immature" birds is significantly lower than that of "adult" ones. Other authors call attention to the great variation observed in immature birds (YU and MARQUARDT, 1974) or point to the sharp rise occurring 2 to 4 weeks before initiation of oviposition which may result in morphological and functional transformation of the oviduct and in an alteration of the calcium metabolism (SENIOR, 1974).

In accordance with our observation the plasma progesterone level demonstrates a gradual increase up to 15th weeks of age, followed by a steep decrease prior to and during maturation. Comparing these results with those

obtained by SENIOR (1974) one can conclude that before maturation there exists an asynchronous shift in estrogen and progesterone levels. The fall of previously consolidated progesterone level coincides with a short term "pre-maturation" peak of estrogen. This fact may point to the antagonistic relations of the two hormones or to their separated regulatory pathways during maturation.

A certain analogy may be revealed during ontogenesis between concentration changes of plasma estrogen in female rats and the variations of progesterone in hens. The high estrogen concentration which is characteristic of immature rats sharply decreases during prepuberty and puberty (DÖHLER and WUTTKE, 1974, 1975). In the domestic hen however where, presumably progesterone (*i.e.* not estrogens) functions as an LH trigger, the progesterone level exhibits variations similar to those exhibited by estrogens in mammals.

According to our investigations no variation of the plasma testosterone level during ontogenesis exists in hens. The same result was obtained by DÖHLER and WUTTKE (1974, 1975) concerning female rats.

These data may suggests indirectly that testosterone does not play a fundamental role in initiating the ovulatory cycle.

The repeated sampling of birds age 15, 21 and 22 weeks permitted us to investigated diurnal variations of plasma testosterone and progesterone prior to and during maturation. We failed to found any diurnal rhythm of the two sexual steroids, and their irregularly fluctuating curves demonstrate high individual variation. Evolution of the ovulatory cycle appears to be able to "synchronize" plasma levels of these hormones, indicating that the so-called interovulatory basic level emerges. This basic level results in a plateau varying only to a small extent in the case of progesterone, while it produces peaks emerging every 8 to 10 hrs, when testosterone is considered (PÉCZELY et al., 1979). Initiation of oviposition is responsible for "preovulatory" peaks originating from the basic level of progesterone and it makes the sinusoid waves of testosterone more irregular, characterized by peaks emerging at the 10 to 12-hr before oviposition.

Summary

Peripheral plasma progesterone and testosterone content of TR-90 hybrid chickens aged between one day 3, 15, 21 and 22 weeks were measured by means of radioimmunoassay (RIA). Plasma progesterone concentration increases till 15th week it show a decrease before and after maturation and reaches the value typical for 15 weeks of age again at the time of laying the first egg. In immature chickens aged 15 to 21 weeks, progesterone exhibits some irregular peaks continuing in a low base line at maturation and producing "pre-ovulatory" surges in chickens aged 22 weeks.

Plasma testosterone level does not show any change from day 1 up to sexual maturation. In chickens of 15 to 21 weeks of age there exist one or two irregular daily peaks while during maturation peaks become more regular and appear at intervals of 8 to 12 hr. Development of ovulatory cycle results in a re-arrangement (a new synchronization) of diurnal changes in plasma progesterone and testosterone level.

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VARIATIONS IN PLASMA LEVEL OF SEXUAL STEROIDS DURING THE OVIPOSITION CYCLE IN LAYING HENS *(GALLUS DOMESTICUS)*

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Ovulation of the first-laid eggs of the clutch occurs early in the morning in *Gallus domesticus*. Protein and egg shell production requires 24.5 hr and the second ovulation of the clutch happens 30–75 min after oviposition. Four or 5 eggs are formed in a run followed by a one-day-long break which introduces the new clutch (FRAPS, 1955). The basic role of LH in eliciting ovulation has been demonstrated in birds by FURR et al. (1973), WILSON and SHARP (1973), LAQUË et al. (1975) and SHABABI et al. (1975). The role of certain ovarial steroids in the mechanism of ovulation has been poorly known, and still represents a controversial question. The ovulation-producing ability of progesterone been long-known (NEHER and FRAPS, 1950; RALPH and FRAPS, 1960). The fact that ovulation regularly ensues 4 to 7 hr after a plasma progesterone peak which coincides with, or seems to precede, the LH peak (ARCOS and OPEL, 1971; CUNNINGHAM and FURR, 1972; FURR et al., 1973; HAYNES et al., 1973; LAQUË et al., 1975; PETERSON and COMMON, 1971; SHABABI et al., 1975) points to the triggering effect of LH. Estrogens block ovulation when implanted into hypothalamic areas (STETSON, 1972) or rather they do not display a pre ovulatory peak (SHABABI et al., 1975; LAQUË et al., 1975). The plasma testosterone level is relatively high in laying hens, and its peak coincides with the pre ovulatory progesterone peak (SHABABI et al., 1975), or appears some two hours before the progesterone and LH peaks (ETCHES and CUNNINGHAM, 1977).

Little evidence is known about simultaneous determination of sexual steroids and about their temporal relations to the ovulatory cycle. That is why we found it justified to investigate the progesterone, testosterone, E_1 and E_2 contents of peripheral plasma in laying hens.

Materials and methods

Five New-Hampshire laying hens, aged one and a half year were used. The birds were fed laying hen feed and water *ad libitum*. 14L—10D photoperiod has been applied throughout, beginning at 6.00 a.m. A 3-ml sample of blood was drawn from each animal by means of a cannula inserted in the v. brachialis, every 3rd or 4th hr for 48 hr. Red blood cells suspended in bird's Ringersolution were re-injected every 8th hr. Ovulation was timed from oviposition. Blood was centrifuged at 3000 rpm, plasma was stored in polyethylene tubes below —20 °C. Sephadex LH-20 chromatography was carried out for simultaneous determination of sexual steroids (MIKHAIL et al., 1970). Recovery

rates of the chromatography were as follows: progesteron: 90%, testosterone: 75%, E_1: 60%, E_2: 45%.

The sexual steroids were determined individually by RIA of the corresponding chromatographic fractions. Progesterone was measured according to ABRAHAM et al. (1971) by means of an antiserum to 11-α-OH-progesterone hemi-succinate-BSA (titre = 1: 4500, B_0 = 50%); testosterone was measured according to JALLAGEAS (1975) with an antiserum to testosterone-hemi-succinate (titre = 1: 8000, B_0 = 45%). In the measurements of E_1 an estrone-hemi-succinate-BSA antiserum (titre = 1: 5000, B_0 = 45%), in that of E_2 an estradiol-hemi-succinate-BSA antiserum (titre = 1: 50 000, B_0 = 40%) was applied. Estrogens were measured according to MIKHAIL et al. (1970). All the antisera had been raised in steep. Progesterone and E_1 antisera by Dr L.-E. EDQVIST (Royal Veterinary College, Uppsala), testosterone and E_2 antisera by Dr P. SHARP (Poultry Research Centre, Edinburgh). Sensitivity and reproducibility of the RIA were checked throughout, its specificity was ascertained by means of cross-reactivity control in pilot experiments. Tritiated samples were counted in a TRICARB 3375 (Packard) liquid scintillation counter.

Results

The sexual steroid level in the plasma of the five laying hens fluctuated strongly during the ovulation cycle. Steroid hormone levels varied in the following ranges: Progesterone = 600—2500 pg/ml, testosterone = 25—320 pg/ml, E_1 = 40—330 pg/ml, E_2 = 25—270 pg/ml.

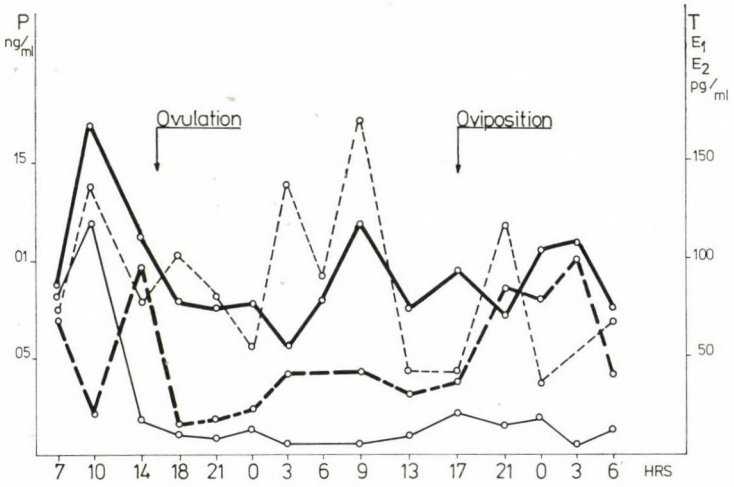

Fig. 1. Fluctuation of plasma level of the sexual steroids in hen no. 1 during the ovulation-oviposition cycle. ▬▬ progesterone; ▬ ▬ ▬ testosterone; — — — E_1; ——— E_2

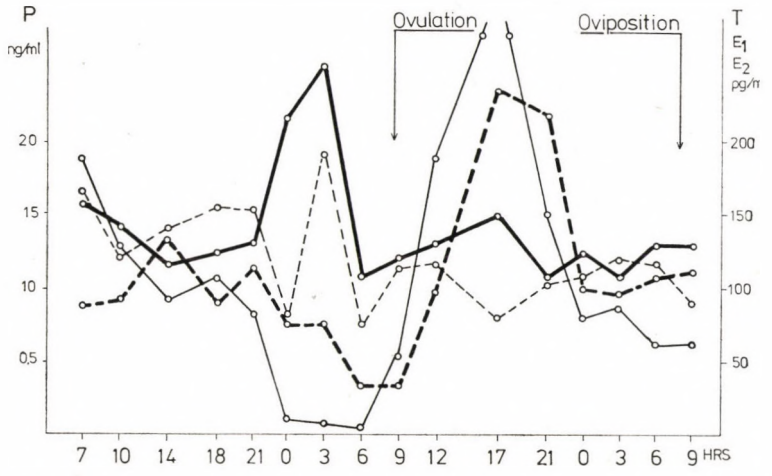

Fig. 2. Fluctuation of plasma level of the sexual steroids in hen no. 2 during the ovulation-oviposition cycle. ▬▬ progesterone; ▬ ▬ ▬ testosterone; — — — E_1; ——— E_2

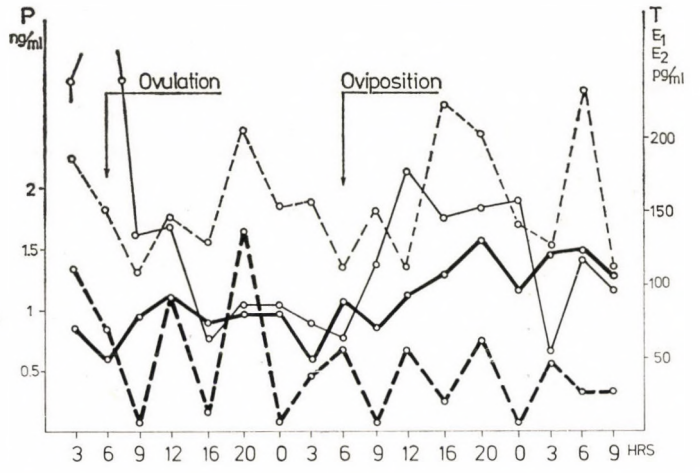

Fig. 3. Variations in plasma level of the sexual steroids in a hen being in interovulatory phase. ▬▬ progesterone; ▬ ▬ ▬ testosterone; — — — E_1; ——— E_2

Progesterone displayd a well-defined peak in the 5th or 6th hr before ovulation, decreasing then considerably and showing only smaller fluctuations (Figs 1 and 2). In the case of animals without ovulation, no peak could be detected in the slightly fluctuating progesterone curves (Fig. 3).
and occasionally these appeared 10—12 hr before oviposition. In certain cases, on the other hand, small peaks appearing every 6th to 8th hr seemed to be characteristic of the testosterone curve (as it is striking in Fig. 3 during the interovulatory phase. In none of the cases could a testosterone peak of the

"pre ovulatory" type be demonstrated. When progesterone exhibited a pre ovulatory peak, testosterone was at a low or very low level (Figs 1—2).

Some irregular peaks of E_1 and E_2 could be observed. Some of these correspond to the preovulatory surge of progesterone or, less frequently, they seemed to appear simultaneously.

E_1 and E_2 peaks may also be evidenced in non-ovulating animals.

Discussion

The results obtained here confirm literary data according to which progesterone exhibits a so-called "pre ovulatory surge", suddenly emerging 4—7 hr before ovulation. The relation of this peak of the ovulatory mechanism has been proved by the slightly fluctuating basic level of progesterone in the interovulatory period without any peak. Similar conclusions were drawn by MASHALY et al. (1976) from experiments in turkeys.

The regulatory functions of esterogens in ovulation are not yet clear. Some investigators (JOHN et al., 1974; SCHRÖCKNADEL and BATOR, 1973) suggest the existence of only one estrogen peak in the laying period which as a rule precedes ovulation by 4—6 hr. Others (PETERSON and COMMON, 1972; LAQUE et al., 1975; SHABABI et al., 1975) detected more than one irregular estrogen peak which could not be interlinked to ovulation. In conformity with our results, E_1 and E_2 may exhibit, asynchronous peak(s) besides peaks synchronous to the progesterone peak. Our results may be interpreted as suggestive of a synergism between estrogens and progesterone in the regulation of ovulation, but not of further interrelations. Bearing in sight the role of estrogens in the protein, lipoid and calcium metabolism of the egg, we may suppose that the non-parallel elevations in E_1 and E_2 concentrations asynchronous to the progesterone are related to metabolic processes of egg formation.

There are only two papers dealing with plasma concentrations of testosterone, or with its possible physiological role, in hens (SHABABI et al., 1975; ETCHES and CUNNINGHAM, 1977).

Testosterone exhibits a pre-ovulatory peak coincident with the peak of progesterone and of estrogens as stated by the above-cited authors. According to them, the joint action of all sexual steroids would trigger LH. ETCHES and CUNNINGHAM (1977) draw the conclusion that testosterone plays a primary role in triggering LH, because its highest level precedes the pre ovulatory LH peak by 2 hr. Data presented here do not support the view of SHABABI et al. (1975). In the case of birds investigated by us testosterone shows low or minimum values in the course of the pre ovulatory progesterone peak. It should be admitted, however, that in two ovulatory cycles one could detect relatively high testosterone levels preceding the pre-ovulatory progesterone surge,

which in turn normally coincides with low TEST levels. The matter in point seems not to be a distinct peak, but only a relatively high value of the fluctuating testosterone level.

We also observed testosterone peaks coinciding with estrogen peaks in the 10th to 12th hr before oviposition. It is a well-known fact that calcium absorption from the jejunum (COMMON et al., 1948) and formation of medullary bone (SIMKISS, 1967) is accelerated if estrogens and androgens act together. One may suggest that elevation in plasma testosterone concentration in the 10th to 12th hr before oviposition, or sometimes coinciding with estrogens, acts upon replenishing of calcium deposits. We intend to investigate these questions in further experiments, both *in vivo* and *in vitro*.

Summary

The concentration of progesterone, testosterone, estrone (E_1) and of 17-β-estradiol (E_2) during ovulation cycle was studied in the blood plasma of laying New Hampshire hens aged 1.5 year. Radioimmunoassay was applied after LH 20 chromatography. The plasma level of progesterone ranges from 600—2500 pg/ml, representing a well-defined peak 5—6 hr before ovulation. Plasma concentration of testosterone was between 25 and 320 pg/ml, negatively correlated with progesterone and gave the highest value 10—12 hr before oviposition, sometimes overlapping the estrogene peak. Ranges for E_1 and E_2 were 40—330 pg/ml and 25—720 pg/ml, respectively. Their several peaks are irregular some of them are simultaneous with the progesterone peaks. While progesterone probably takes a prominent role in the regulation of ovulation, testosterone and estrogens are supposed to exert a regulatory effect on the metabolic process of egg-formation.

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ABNORMAL ELECTROCARDIOGRAMS IN LEUCOTIC CATTLE

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Bovine leucosis frequently gives rise to neoplastic lymphoid cell proliferation in the heart, especially in the right auricle.

A survey of postmortem findings has revealed that cardiac involvement is next in frequency to lymph node alterations in bovine leucosis (LOPPNOW et al., 1971).

Electrocardiographic studies have made possible the recording of the action potential of the functioning heart. Opinions have been divergent on the value of electrocardiography in leucosis diagnostics; certain authors have regarded it as fully conclusive (KARGE and WERNER, 1963), while others have questioned its importance (SANDER, 1968; CAKALA, 1972).

The true cause of leucotic heart lesions is poorly understood. Some investigators believe that the subepicardial reticulum cells are particularly liable to proliferation under leucotic conditions, while others (KARDEVÁN, 1976) have suggested a lymphogenic spread of the process from the anterior mediastinal lymph nodes to the atrial wall.

In earlier diagnostic studies, we often found neoplastic myocardial lesions in the heart of leucotic cattle. Cell proliferation was as a rule grossly apparent in such cases. It therefore seemed worth-while to pursue in great detail the diagnostic value of ECGs in bovine leucosis.

Materials and methods

About 200 bovines were examined by electrocardiography. The greater part of the tracings were utilized for methodical purposes. The study reported in this paper covered 47 animals, of which 20 were healthy, 15 were clinically ill in neoplastic leucosis, and 12 were in the preneoplastic stage of the disease, having exhibited persistent lymphocytosis without symptoms. Tracings from these animals were used for comparative evaluation of ECG changes characteristic of the disease.

The diagnosis of neoplastic leucosis was based on clinical evidence of neoplastic changes in all body regions, with corresponding histological findings. Clinically, the leucotic animals showed abnormal palpability of certain lymph nodes, haemolymph nodes, and frequently also symptoms indicative of heart

involvement, such as dyspnoea, cachexy, poor general condition and depressed performance. Occasionally signs of chronic cardiac insufficiency were also present (upper thoracal oedema, dilatation of the jugular veins, injected vessels in the sclera, high pulse rate, *etc.*).

The diagnosis, reneoplastic leucosis, was based on the haematological evidence of persistent lymphocytosis in tests performed not earlier than six weeks before the ECG examinations and the absence of clinical symptoms.

Healthy bovines were included in the study as control group.

The animals were not subgrouped with regard to age, breed or sex, for the juvenile and adult (enzootic) forms of the disease differed neither clinically nor pathologically. The youngest animal studied was a four-week-old calf, the oldest one was a 10-year old cow. As to breed, 40 and 60% of the leucotic animals belonged to the black-and-white Lowland and Hungarian red-pied types, 90 and 10% of the preleucotic cases were black-and-white Lowland and Danish Red, and the healthy animals represented the above Lowland and Hungarian types half-by-half.

ECG recording was performed in a room specially adapted to the purpose (with electric insulation and soundproof). Before recording, the animals were allowed to adapt to the environment for 15—20 min. As a rule no sedative treatment was made; three healthy animals had been injected i.m. with 1.0—1.5 ml Rompun inj. (Bayer) to test its effect on the ECG record. Conform to the observations of others, the tranquillizer depressed heart rate and enhanced vagotony (the data are not included in the Tables).

A direct-recording, three-lead electrocardiograph (9400 THS Multiscripto, -Hellige) was employed throughout. All tracings were taken at 25 mm/sec. strip velocity; a 10 mm action potential corresponded to 1 mV.

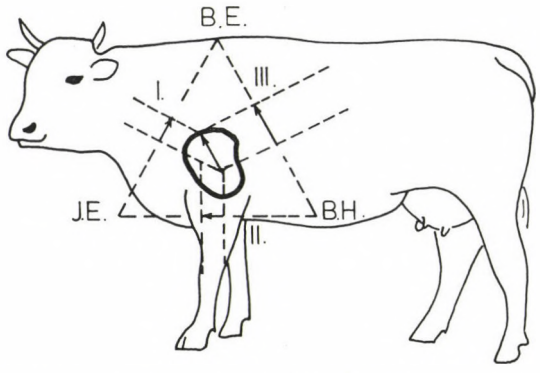

Fig. 1. Two-dimensional depiction of the sagittal leads I, II and III. BE, left anterior limb electrode; JE, right anterior limb electrode; BH, left posterior limb electrode

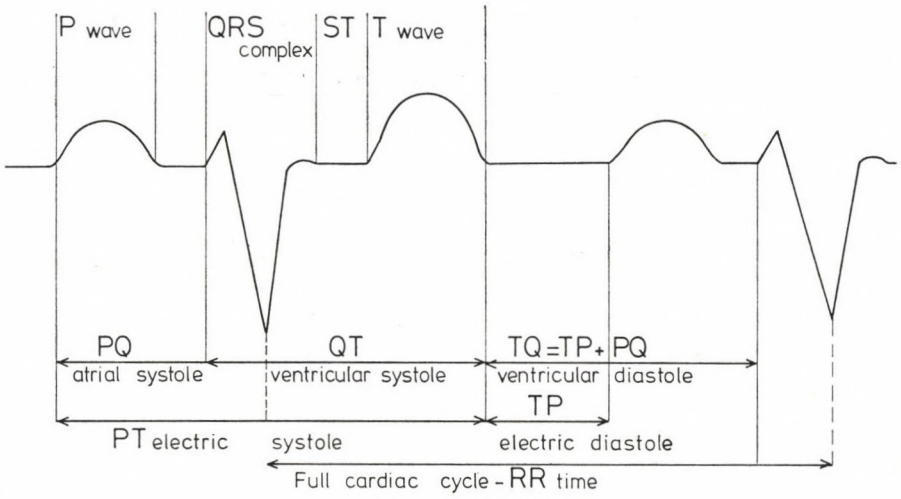

Fig. 2. Components of sagittal tracing in lead III. These components show exclusively the cardiac electric events

Before application of the electrodes the skin was shaved, de-fatted with a mixture of ether and ethanol, soaked in saline, and finally covered with Hellige's "electrodence" ointment. The electrodes were fixed to 4—8 mm long longitudinal skin folds with large pincers, always in the same position.

Several leads were tested in preliminary studies to find the one best-suited for electrocardiography in cattle. As in large animals, including cattle, the Einthoven triangle is not equilateral, but right-angled, the leads employed in human electrocardiography are not fully conclusive.

The sagittal lead employed by us was established as follows: the left anterior limb electrode was fixed in the region of the withers, at the imaginary prolongation of the vertical limb axis, the right anterior limb electrode at the caudal end of the dewlap; the left posterior limb electrode was applied on the left popliteal fold, in the original place of the right posterior neutral limb electrode (Fig. 1). The tracing obtained from the sagittal lead is shown in Fig. 2.

It should be emphasized that an appropriate local preparation of the skin plays a decisive role in the quality of tracings, for it reduces the blocking effect of the skin on conduction of the action potential. The pincer-fixed electrodes used by us were equivalent in efficiency to the gauze-wrapped, saline-soaked appliances (SPÖRRI, 1954; SANDER, 1968; and many others), as well as to subcutaneous needle electrodes (KARGE and WERNER, 1963) employed by others. We agree with other investigators that the standard limb leads furnish only low-voltage, low-amplitude tracings.

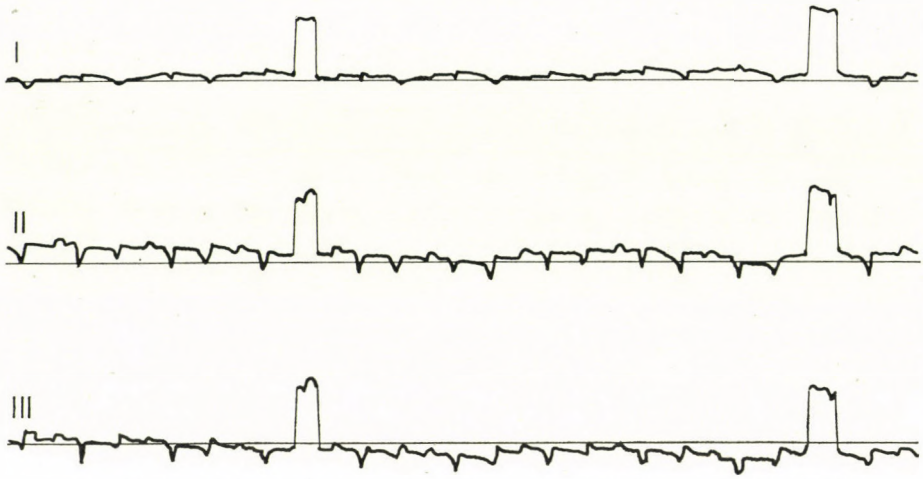

Fig. 3. Tracing on normal cow from the standard (limb) leads I, II and III

Fig. 4. Sagittal tracing from the same normal cow as in Fig. 3

Results

The tracings obtained from the standard (limb) and sagittal leads are compared in Figs 3 and 4. Table I shows the clinical and pathological findings along with the evaluation based on the sagittal tracings. Table II shows the quotients PQ/QT (atrial-ventricular systole), QT/TQ (ventricular systole/ventricular diastole) and PT/TP (electric systole/electric diastole), the QRS complex, ST interval and systolic index % $\left(\text{SI } \% = \frac{\text{QT} \cdot 100}{\text{RR}}\right)$, in terms of mean values ($\bar{x}$) with standard error of the mean ($s\bar{x}$) and variation coefficient (%).

Table I

Diagnostic evaluation of cattle diseased in neoplastic leucosis

Serial no.	Designation (name of) animal	Breed	Age (year or month)	Date of examination	Haematologically positive since	Clinical Pathological change in body	heart	in body	heart	Pulse rate (min.)	ECG evaluation from saggittal leads
1	41 August	B—W	9	18.1.1974	1969	+	—	+	Ma: — Mi +	70	C, *D*, *E*, H_I, I
2	209 Cidrus	B—W	8	2.12.1975	1970	+	+	+	+	90	D, F, *G*
3	498 Alak	B—W	5	5.9.1975	1973	+ rect.	—	+	+	80	A, B, *C*, *D*, E, F, H_{II}, *I*
4	15 Dóra	B—W	7	20.2.1976	1973	+	+	+	+	80	B_I, C, D, *E*, *F*, H_I
5	6258 Hasszana	B—W	5	23.6.1976	neg.	+ rect.	—	+	—	70	D, E, F, G_{II}
6	141 Obur	B—W	11	2.12.1975	neg.	+	+	+	+	120	*A*, *B*, D, *E*, F_I, *G*, H_I
7	5797 Datolya	HRP	6	3.4.1974	1975	+	—	+	—	60	B, C, E, *G*, E
8	5032 Malvin	HRP	7	21.5.1975	1973	+ rect.	—	+	+	90	C, D, H_{II}, *I*
9	22/4078 Elégia	HRP	13	10.2.1976	1972	+	—	+	+	60	E, D
10	19/116 Ajándék	HRP	3	6.2.1976	neg.	+	—	+	+	90	D, E, G_I, III, I
11	559170	HRP	3.5	11.11.1976	neg.	+ rect.	+	+	+	80	D, F_{II}, H_{II}
12	4175 Démon	HRP	13	10.9.1976	1971	+	+	+	+	90	*A*, F_{II}, G_{II}, H_{II}
13	578351	HRP	8 months	3.9.1976	neg.	+	+	+	+	100	*F*, F, H_{II}, *I*
14	I. K.	HRP	1 month	21.7.1976	neg.	+	+	+	+	90	*F*, H_I
15	555799	HRP	1 year	24.2.1974	neg.	+	+	+	+	80	C, D, E, F, H_I

Symbols used: B—W, Black-and-White Lowland; HRP, Hungarian Red Pied; rect., lesion also found at rectal examination; Ma, gross lesions; Mi, microscopic lesion; +, severe change (cursive ECG changes were also severe); A, sinus arrhythmia, extrasystole; B, auricular fibrillation; C, PQ/QT (auricular/ventricular systole) quotient abnormal; D, QT/TQ (ventricular systole/ventricular diastole) abnormal; E, PT/TP (electric systole/electric diastole) abnormal; F, QRS abnormal; G, ST interval abnormal; H, low voltage (in lead H_I, H_{II} or H_{III}); I,high systolic index %.

Table II

Data of tracings from the sagittal leads

Designation: ECG parameters:		C PQ/QT	D QT/TQ	E PT/TP	F QRS	G ST	I Systolic index %
		Lead I					
Neoplastic leucosis	x	0.53	*0.80*	*2.72*	0.08	0.20	*46.6*
n = 15	sx	0.042	0.040	0.004	0.007	0.20	7.8
		±8.0	±5.0	±4.3	±9.4	±4.55	±17
Preneoplastic leucosis	v	0.59	*0.82*	*3.04*	0.073	0.18	*44.8*
n = 12		0.029	0.050	0.079	0.004	0.008	7.3
		±4.9	±6.06	±1.5	±6.60	±4.50	±18.0
Normal	v	0.57	0.71	*1.82*	0.08	0.20	*40.6*
n = 20	v	0.023	0.035	0.022	0.002	0.008	5.2
		±4.12	±4.88	±1.1	±3.48	±4.20	±11.5
		Lead II					
Neoplastic leucosis		0.58	*0.84*	2.93	0.06	0.23	47.4
n = 15		0.025	0.048	1.078	0.006	0.011	7.9
		±4.31	±5.70	±4.05	±9.40	±4.55	±17.0
Preneoplastic leucosis		0.59	*0.83*	*2.95*	0.07	0.20	*44.8*
n = 12		0.019	0.051	0.076	0.007	0.012	7.3
		±3.18	±6.13	±1.66	±9.93	±6.03	⊥18.0
Normal		0.57	*0.69*	*1.81*	0.07	0.22	*40.6*
n = 20		0.015	0.041	0.050	0.006	0.008	5.2
		±2.70	±6.00	±8.3	±8.00	±3.50	±15.5
		Lead III					
Neoplastic leucosis		0.55	*0.86*	*2.79*	0.08	0.20	*47.7*
n = 15		0.027	0.042	0.008	0.005	0.012	7.8
		±4.90	±4.88	±2.40	±6.37	±5.90	±17.0
Preneoplastic leucosis		0.58	*0.83*	2.95	0.075	02.0	*44.8*
n = 12		0.020	0.44	0.060	0.055	0.011	7.3
		±3.49	±6.45	±1.77	±7.37	±5.75	±18.0
Normal		0.55	*0.72*	*1.90*	0.08	0.22	*40.6*
n = 20		0.015	0.033	0.050	0.002	0.008	5.2
		±2.76	±4.62	±1.20	±2.30	±3.60	±11.5

The cursive print indicates significance at the $P < 5\%$ level.

Discussion

In this paper exclusively the sagittal tracings are evaluated, for — according to our own experience — this technique has several advantages over the standard (limb) and other leads (ALFREDSON and SYKES, 1940; BROOIJMANS, 1954; CORTICELLI, 1954; GABRASCHANSCKY, 1954; HLAVKA, 1918; HOLMES, 1960; KARGE and WERNER, 1963; NÖRR, 1921; SANDER, 1968; SELLERS et al., 1958; SPÖRRI, 1954). It produces sufficiently large and comparable tracings, and makes possible the insertion of the heart's electric axis

into an equilateral triangle with a vertex pointing upward. Sagittal tracings on healthy animals have consistently shown the highest amplitudes in leads II and III; the R wave was negative in lead III, while nearly always positive in lead I. It is usually negative, occasionally positive, in lead II. This corresponds essentially to SANDER's (1968) type-I bovine ECG. In the sagittal tracings R_{III} is equal to $R_I + R_{II}$.

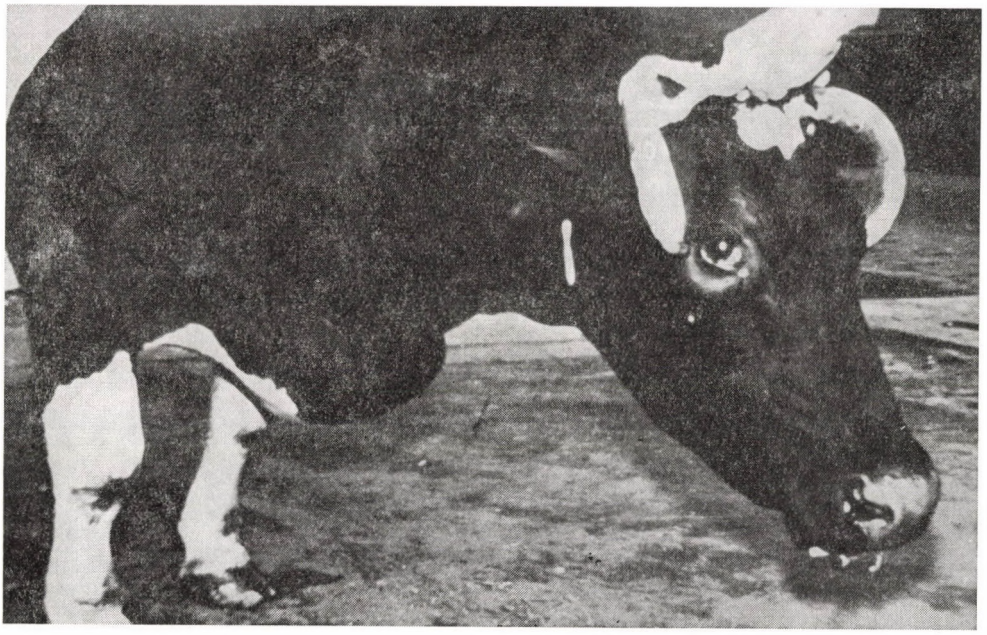

Fig. 5. Oedematous thoracal infiltration in cow 141-Obur. Cardiac insufficiency due to involvement of the heart by neoplastic leucosis

SANDER (1968) and CAKALA (1972) suggest that cardiac leucosis, *i.e.* the severity of heart involvement in leucosis, rarely shows an unequivocal correlation with ECG abnormalities. KARGE and WERNER (1963) observed consistent appearance of abnormal parameters in ECG tracings from leucotic cattle, and have regarded prolongation of the PQ interval, appearance of broad, flat R waves and increase of the systolic index % as diagnostically relevant indications of leucosis.

The data presented in Table I indicate that sinus arrhythmia, extrasystole and auricular fibrillation frequently appear in the ECG of leucotic cattle. The abnormalities designated with the letters A, B, C, D, E, F, G, H and I were variably present. Table II shows that the quotient PQ/QT was even in all three groups, while the value of QT/TQ was high relative to normal both in the leucotic and preleucotic groups. Also the value of the quotient PT/TP,

Table III

Values for P, R, and T waves in sagittal tracings (means and limits)

Waves	Lead I		Lead II		Lead III	
	sec	mV	sec	mV	sec	mV
		Neoplastic leucosis n = 15				
P	—	—	0.08 0.04—0.12	0.16 0.1—0.3	0.07 0.04—0.12	0.14 0.1—0.2
R	0.08 0.04—0.12	*0.40* 0.15—0.75	0.06 0.04—0.08	*0.60* 0.2—1.8	0.09 0.04—0.12	*0.77* 05.—1.8
T	0.08 0.04—0.12	0.20 0.10—0.30	0.09 0.08—0.12	0.35 0.15—0.6	0.08 0.06—0.12	0.32 0.2—0.6
		Preneoplastic leucosis n = 12				
P	0.05 0.04—0.08	0.11 0.1—0.15	0.07 0.04—0.12	0.16 0.1—0.3	0.07 0.04—0.08	0.15 0.1—0.2
R	0.07 0.02—0.08	*0.45* 0.1—1.1	0.06 0.04—0.08	*0.62* 0.1—0.8	0.07 0.04—0.08	*0.70* 0.4—1.2
T	0.08 0.04—0.12	0.30 0.1—0.7	0.08 0.06—0.12	0.43 0.2—1.2	0.08 0.06—0.08	0.35 0.15—0.6
		Normal n = 20				
P	0.06 0.04—0.08	0.10 0.10—	0.06 0.04—0.08	0.15 0.10—0.20	0.07 0.04—0.08	0.13 0.10—0.20
R	0.08 0.04—0.12	*0.55* 02.—1.0	0.06 0.04—0.08	*0.50* 0.1—1.40	0.07 0.04—0.08	*0.66* 03.—1.2
T	0.08 0.06—0.12	0.32 0.1—0.7	0.08 0.04—0.12	0.32 0.1—0.8	0.08 0.06—0.12	0.36 0.2—0.7

For explanation see Table. II.

which represents the prolongation of the electric systole, was high in both diseased groups. The systolic index % increased over the control in both neoplastic and preneoplastic leucosis. High PT/TP and high systolic index are complementary to one another in reinforcing the diagnosis. It appears that prolongation of the electric systole was the most consistent ECG change in leucotic cattle. The R wave exhibited consistent alterations in leads I and III (Table III); it had a low amplitude and long duration, occasionally with duplication in lead I; it increased in lead II and, conspicuously, in lead III. Isoelectric Q and S waves frequently appeared in all three leads from leucotic and preleucotic cattle. A moderate to pronounced depression of the ST interval — in the latter case below the isoelectric axis — was found to occur in neoplastic leucosis.

Some animals with neoplastic leucosis showed distinct signs of cardiac insufficiency. At physical examination an enormous pasty swelling was palpable like in pericarditis (Fig. 3), and postmortem examination revealed presence of neoplastic lymphoproliferative lesions on the epicardium, in the external atrial muscle layer and within the auricles themselves. The heart muscle itself was scarcely involved by the destructive proliferation of lymphoid cells, only peri-

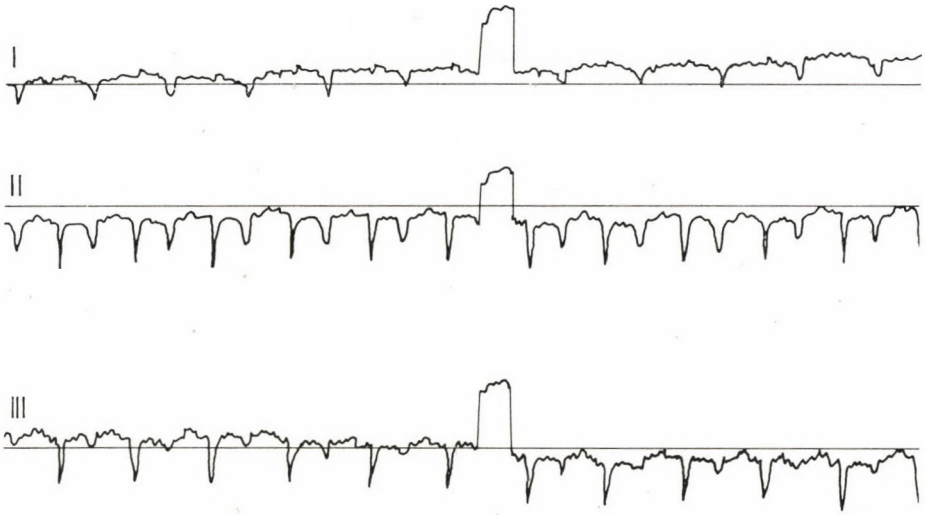

Fig. 6. Sagittal tracing from cow 141-Obur; it is abnormal in lead I, and almost normal in leads II and III

cardial tumours occursed. The ECG tracing was nevertheless characteristic of leucosis (Fig. 6). In one case (Fig. 7) the abnormal ECG was due to severe neoplastic alterations of the myocardium (Fig. 8). According to SANDER (1968) the bovine heart muscle comprises within the system of fibres a cellular structure, and the Purkinje fibres do not directly and wholly join the working muscles, but often pass across these and end on the epicardium. This can account for the very short duration of the QRS complex and for ventricular depolarization, which accord well with the tracing judged by us as abnormal on the basis of prolonged QT-interval, and increased duration of QT/TQ, PT/TP and systolic index %. The abnormal tracing often exhibits isoelectric Q and S waves, low voltage of the QRS complex in one of the leads and depression of the ST interval beneath the isoelectric axis. According to ALFREDSON and SYKES (1940), and SANDER (1968), ST depression is less indicative of pathological condition in the bovine than in the carnivores or the horse. Follow-up of the appearance and variations of the ST interval may open up new scopes in the ECG diagnostics of bovine leucosis.

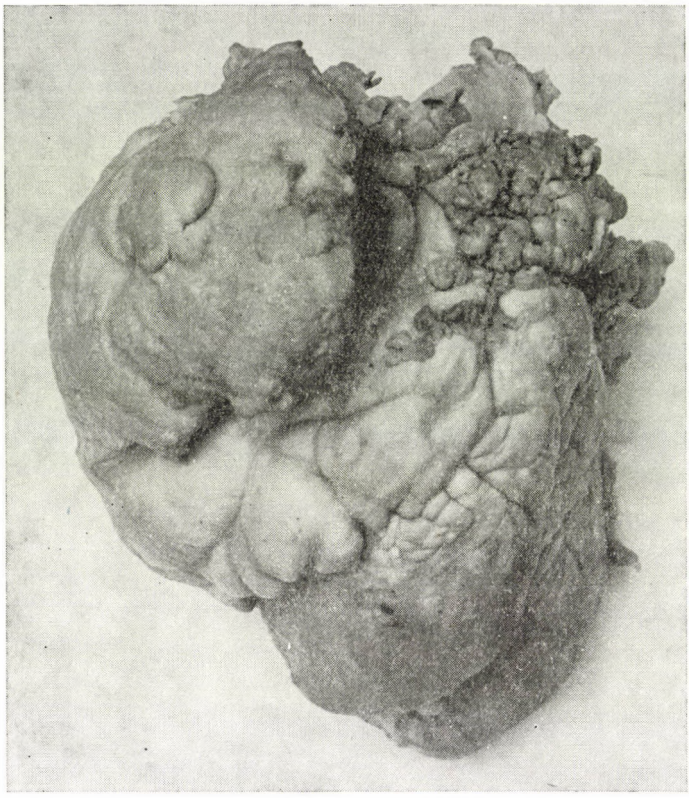

Fig. 7. Neoplastic alteration of the heart in cow 498-Alak, diseased in neoplastic leucosis

Fig. 8.

Summary

Electrocardiographic studies were performed with the sagittal lead technique on 15 cattle, with neoplastic and 12 with preneoplastic leucosis (persistent lymphocytosis), and on 20 healthy bovines.

Tracings from the sagittal lead appear to be comparable and suitable for diagnostic evaluation.

Tracings on cattle with neoplastic leucosis exhibited the following changes: (a) auricular fibrillation, (b) prolongation of the electric systole, and rise of the QT/TQ, PT/TP quotients and of the systolic index, (c) duplication of the QRS complex and predominance of the R wave in leads II and III, (d) frequent occurrence of isoelectric Q and S waves.

The same changes, although in a less pronounced form, appeared in part of the tracings from preleucotic cattle.

The leucotic involvement of the heart was not always portrayed in the ECG; thus the diagnostic value of electrocardiography is limited in bovine leucosis.

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The Handbook of British Mammals. Edited by G. B. CORBET and H. N. SOUTHERN. Second Edition. Published for the Mammal Society by Blackwell Scientific Publications, Oxford—London—Edinburgh—Melbourne. 1977. First published in 1944, reprinted in 1965. Price: £ 9.75.

The attractively bound volume includes the description of British mammals on 520 pages, under the systematic classification also used in Hungary. The great interest aroused by the first edition of the Handbook necessitated its re-printing within one year, in 1965, and the reprinted volumes, too, disappeared soon from the bookstores. A second completed edition, which includes the newer research observations disclosed in the meantime, was published in 1977.

Round 100 mammalian species are described; the domesticated or nationalized species are also discussed briefly. A short historical review is devoted to extinct species — to each in its systematic position — among others to the brown bear, exterminated from Great Britain by hunting and trapping for bear-baiting.

An introductory List of Species, in which the latter are ordered into the known taxonomic categories (Order, Family, Genus) is followed by detailed description of the species discussed. A survey of the specific features making possible recognition is followed by a detailed analysis of the external appearance of males and females, especially in those species in which sexual dimorphism is pronounced. Clear and concise Tables are presented, where necessary, on the measurements of males and females. Separate subchapters deal with subspecies and/or variants. Black-and-white maps facilitate the easy orientation of readers with faunistic interest in the geographical distribution of the mammalian species and sub-species discussed.

Each chapter includes the newest research observations available on the behaviour, habits, diet and reproduction of the species.

It is a great merit of the bromatological descriptions that, by including the percentuar distribution of the types of feed — plant or animal — consumed by the species, they show by themselves wheather the latter is useful or a pest.

In several cases the reproduction biological data presented close long-existing gaps of knowledge on certain details in the sexual cycle of e.g. the bat (Chiroptera) and some Mustelidae species.

Predator "enemies" of the species and the main causes of mortality in populations are specified wherever relevant, or known.

The greatest appeal to the veterinary reader is nonetheless the information presented on the diseases as will as external and internal parasites of the species covered in the Handbook.

Last but not least the structure of the Handbook deserves words of appreciation: the consistent logical order of the chapters and presentations greatly facilitates comprehension, and also correlations between related species.

The excellent photographic and clear-cut graphical illustrations greatly add to the value of the book.

M. JANISCH

INDEX

Bacteriologia

PESTI, L. and SEMJÉN, G.: *Escherichia coli* Vaccine for Prevention of Neonatal Enteric Colibacillosis in Pigs 1

PESTI, L. and SEMJÉN, G.: *E. coli* Heat-Labile (LT) Antitoxin Levels in Pigs and in *E. coli*-Vaccinated Sows 12

Virologia

Шврчек, Ш., Александер, Р., Вртяк, О. Я., Мачичка, О., Грулих, И. и Ондрейка, Р.: Изоляция вируса бешества из хомяка обыкновенного (Cricetus cricetus) в Чесословакии 21

Шврчек, Ш., Вртяк, О. Я., Герлахов, Д., Голы, Л., Анталик, Д., Александер, Р. и Ондрейка, Р.: Пероральна яинфекци явирусом бешенства лисицы и кошки домашней 27

Шврчек, Ш., Вртяк, О. Я., Селимов, М. А., Аксенова, Т. А., Герлахов, Д., Голы, Л., Дюран, А. и Байова, В.: Экспериментальные предпосылки и техника пероральной антирабической иммунизации диких плотоядных. I. Предварительные опыты на лабораторных животных 33

Шврчек, Ш. и Вртяк, О. Я.: Модификация количественного метода ИПВЭ для определения иммуногенной активности антирабических вакцин в опыте на мышах 43

BELÁK, S.: Properties of Bovine Adenoviruses 47

Parasitologia

JUHÁSZ, S., GÁLFI, P. and MOLNÁR, K.: Data on the Proteolytic Enzyme System of *Lernaea cyprinacea* 57

Physiologia

SZEGEDI, B., JUHÁSZ, B., SZELÉNYI-GALÁNTAI, Marianna, JÉCSAI, Jolán and TELEKI, Mária: Experimental Studies on Early-Weaned Lambs. I. Development of Gastric Digestion and Metabolic Changes during Weaning 71

SZELÉNYI-GALÁNTAI, Marianna, JÉCSAI, Jolán, JUHÁSZ, B., SZEGEDI, B. and TELEKI, Mária: Experimental Studies on Early-Weaned lambs. II. Nitrogen and Amino Acid Metabolism 85

MURAY, T., PETHES, GY. and PÉCZELY, P.: Changes of Plasma Progesterone and Testosterone Level in Chicken (*Gallus domesticus*) from the First Day of Life to the Beginning of Oviposition 97

PÉCZELY, P., PETHES, GY., SZELÉNYI, Z. and MURAY, T.: Variations in Plasma Level of Sexual Steroids during the Oviposition Cycle in Laying Hens (*Gallus domesticus*) 103

HORVÁTH, Z. and SZEKERES, T.: Abnormal Electrocardiograms in Leucotic Cattle 109

Bibliographia 120

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РЕЗЮМЕ

ВАКЦИНА ИЗ КИШЕЧНОЙ ПАЛОЧКИ ПРОТИВ КОЛИБАКТЕРИОЗА НОВОРОЖДЕННЫХ ПОРОСЯТ

Л. ПЕШТИ и Г. ШЕМЙЕН

По сравнению с контрольными к минимальному удалось снизить заболеваемость колибактериозным поносом среди подсосных поросят свиноматок, привитых в супоросный период инактивированной 0,2%-ным формалином, адъювантной вакциной из кищечной палочки, содержащей энтеротоксины LT и ST.

Наступивший после прививки пассивный иммунитет в силу его антитоксического характера независим от серологического типа вызывающих болезнь штаммов, то есть защищает он как против гомологических, так и гетерологических таковых. Выработка адекватного гетерологического иммунологического ответа доказана собственной антисывороткой в пробе с кишечной петлей. Защитительная мощность антисыворотки, полученной при помощи вакцины в пробе с кишечной петлей независима от количества микробных телец кишечной палочки и микрофлоры петли.

Антисыворотка тормозит петлю расширяющее действие бульйонной культуры кишечной палочки и нейтрализирует не только энтеротоксин LT, но в меньшей степени и энтеротоксин ST. Формалин превращает в вакцине налицевствующие энтеротоксины LT и ST в токсоид.

ТИТРИРОВАНИЕ АНТИТЕЛ ПРОТИВ ТЕПЛОЧУВСТВИТЕЛЬНОГО (LT) ЭНТЕРОТОКСИНА КИШЕЧНОЙ ПАЛОЧКИ В СЫВОРОТКЕ И МОЛОКЕ СВИННОМАТОК, ПРИВИТЫХ И НЕПРИВИТЫХ ВАКЦИНОЙ ИЗ КИШЕЧНОЙ ПАЛОЧКИ

Л. ПЕШТИ и Г. ШЕМЙЕН

С малым видоизменением авторами применялся метод титрирования пастереллезного антитоксина по Касаи в Буровс для определения в молоке и сыворотке титра антитоксина LT против кишечной палочки. При изучении сывороточных пар от свиноматок, содержавшихся в крупнохозяйственных условиях и привитых вакциной из кишечной палочки обнаружено, что в 1 мл-е сывороток до прививки в среднем имеется 229 Е антитоксина LT, а после прививки — 1116 Е антитоксина LT. Таким образом у привитых свиноматок основной титр антитоксина повысился в 5 раз. Титр гипериммунной антисыворотки LT равнялся 3528 антитоксическим единицам. В силу прививки вакциной из кишечной палочки по сравнению с непривитыми свиноматками в молозиве привитых таковых количество LT нейтрализирующих антител повысилось в 5 – 6 раз. С возрастом свиней начальные низкие титры антитоксина LT в сыворотке повышаются. Антисыворотка, лишенная антител ОК, не потеряла токсин LT нейтрализирующего действия. Согласно авторам в защитном действии вакцины важнейшую роль играет антиэнтеротоксический иммунитет.

ISOLATION OF RABIES VIRUS FROM COMMON HAMSTERS INDIGENOUS IN EASTERN SLOVAKIA

By

Š. Švrček, R. Alexander, O. J. Vrtiak, O. Mačička, I. Grulich and P. Ondrejka

Carcases of a total of 283 hamsters collected around seven communal settlements in Eastern Slovakia in 1972 and 1973 were examined for presence of rabies virus by direct immunofluorescence test and biological test in suckling mice. Three virus strains (3 O, 7 E, 9 E) were isolated from suckling mice. No virus was detected in any of 196 mole carcases collected around two communal settlements in the same region.

The isolates were identified as rabies virus strains by the usual criteria (direct immunofluorescence, presence of Babes-Negri type inclusions and neutralization index).

RESPONSE OF THE FOX AND CAT TO ORAL INFECTION WITH RABIES VIRUS

By

Š. Švrček, O. J. Vrtiak, D. Gerlachov, L. Holý, D. Alexander, D. Antalik and P. Ondrejka

Provocation of clinical rabies in wild fox (Vulpes vulpes) and domestic cat by oral infection with the virus, and appearance of lyssa-antigen in the cornea of the infected animals is reported for the first time in the world literature. The cornea test, the examination of impression smears from inguinal lymph nodes seem to furnish valuable complementary tests in support of diagnostic evidence.

PREREQUISITES AND TECHNIQUE OF THE ORAL IMMUNIZATION OF WILD CARNIVORES AGAINST RABIES. I. PRELIMINARY EXPERIMENTS IN LABORATORY ANIMALS

By

Š. Švrček, O. J. Vrtiak, M. A. Selimov, T. A. Aksenova, A. Gerlakhov, L. Holý, A. Djuran and B. Bájová

The prerequisites of succesful oral immunization against rabies were studied in laboratory animals (guinea-pig, rabbit). This original method of rabies prevention in wild carnivores was described first in 1970 (Vrtiak el al.).

In experiments on oral immunization of guinea-pigs, a live vaccine prepared from the strain Vnukovo-32 proved to be the most immunogenic. Immunogenicity assays have led to similar conclusions. An inactivated vaccine prepared from the same strain failed to develop antigenic activity in rabbits. An appropriately attenuated vaccine strain of the Vnukovo-32 virus seems to be suitable for the preparation of a commercial oral vaccine.

IMMUNOGENICITY TESTING OF RABIES VACCINES BY A MODIFIED QUANTITATIVE ASSAY

By

Š. Švrček and O. J. Vrtiak

Modification of a quantitative assay procedure, originally developed in the Institute of Poliomyelitis and Virus Encephalitides (Moscow. USSR), is described. The modified procedure is strictly quantitative, easily reproducible, and it makes possible a natural-like, peripheral infection of the experimentally immunized animals. The working principle is that all test vaccines are applied subcutaneously, on a single occasion, and CVS fix virus is used for challenge.

СВОЙСТВА АДЕНОВИРУСОВ ОВЕЦ

Ш. БЕЛАК

Изучением заболеваний дыхательных путей и желудочно-кишечнего тракта, появившихся после массового откорма ягнят в крупных хозяйствах, изолированы реовирусы, вирус параинфлюэнцы-3 и аденовирусы. Резюмируя данные своей кандидатской диссертации автор докладывает об изоляции аденовирусов и приводит доказательства об их патогенности. Подробно ознакомляет с физическими свойствами вирусов, в том числе с морфологией, размерами и плавательной плотностью вириона. Резюмирует данные по резистентности вирусов к физико-химическим вмешательствам, в том числе к дезинфицирующим средствам. Изучает нуклеиновую кислоту и структурные протеины вириснов. Докладывает о клетки повреждающей способности разных штаммов, цикле репликации на стойких и вращающихся средах в разного возраста и происхождения клеточных культурах. За репликацией следит электронным микроскопом и путем иммунофлюоресценции. Изучаются гемагглютинационные свойства овечьих и некоторых бычьих аденовирусов и обнаруживает маркер для дифференциации вирусов этих двух видов. В сыворотке овец обнаруживает такие антитела, которые реагируют только с антигеном второй подгруппы аденовирусов крупного рогатого скота. Детальное изучение свойств овечьих аденовирусов является основением разработки мер специфической борьбы с заболеваниями ягнят, вызванными этим патогенным началом.

О СИСТЕМЕ ПРОТЕОЛИТИЧЕСКИХ ЭНЗИМОВ У LERNAEA CYPRINACEA

Ш. ЮХАС, П. ПАЛФИ и К. МОЛНАР

Экстракты из Lernaea cyprinacea на основании расщепления гемоглобина, казеина и желатины показывают протеолитическую энзимную активность. При помощи субстратов солянокислый N-α-бензоил-L-аргинин этил эстер и солянокислый N-α-толуен-4-сульфонил-L-аргинин метил эстер наблюдается сильная эстеролитическая активность, при помощи субстрата солянокислый N-α-бензоил-DL-аргинин-4-нитроанилид — слабая амилазная активность. Экстракт не расщепляет N-бензоил-L-тирозин этил эестер. На столбе Сефадекс Г-75 экстракт авторы прочистили и получили только одну фракцию молекулярного веса 14 000. В буферах tris фосфатном и боратном максимальная энзимная активность отмечалась при pH 9. Авторы определили константу Михаелиса энзима (6,810^{-6}M) и его оптимальную температуру (70°C). Ионы кальция, магния и цинка, дальше р-хлоро-ртутно-бензойная кислота и рыбная сыворотка (трипсин ингибитор) не тормозили активности экстракта, ионы кобальта и марганца усиливали его активность, тогда как натриева соль этилендиаминтетра-уксусной кислоты, диизопропилфторофосфат, 1-хлоро-3-тосиламидо-7-амино-2-гептанон, трипсин ингибитор сои и трипсин-ингибитор свиной аскариды тормозили его активность. Энзим, согласно авторам, подобен трипсину.

ЭКСПЕРИМЕНТЫ ПО РАННЕЙ ОТБИВКЕ ИСКУССТВЕННО ВЫРАЩЕННЫХ ЯГНЯТ. I. ФОРМИРОВАНИЕ ЖЕЛУДОЧНОГО ПЕРЕВАРИВАНИЯ У ЯГНЯТ И ИЗМЕНЕНИЕ ОБМЕНА ВЕЩЕСТВ ПОСЛЕ ОТБИВКИ

Б. СЕГЕДИ, Б. ЮХАС, МАРИАННА СЕЛЕНИ-ГАЛАНТАИ, ЙОЛАН ЕЧАИ и МАРИЯ ТЕЛЕКИ

У искусственно выращенных ягнят венгерского мериноса в возрасте 4 – 38 дней изучали функцию сычуга и формирование деятельности рубца и на основании разных параметров крови следили за изменениями обмена веществ. Применяя разную стадийную технологию поения изучали действие отбивки на переваре ние и обмен веществ.

Наблюдения показали, что при стадийном поении ягнят развивающийся рубец тоже наполнился заменителем молока, ибо его количество при одном поении превосходило объем сычуга. От заменителя молока наступил в сычуге резкий сдвиг в pH, концентрации кислоты и хлорида. Вероятно, что это тормозило установление соответствующей микрофлоры и микрофауны в нем и развитие бродильных процессов, что при отбивке развивающийся в рубце механизм переваривания дополняет переваривание в сычуге, поэтому о снабжении соответствующими питательными веществами последнего необходимо заботиться, пока в рубце переварение не становится характерным для взрослой овцы.

Сокращение количества заменителя молока в рационе вело к повышению поедаемости грубого корма. Передозировка или внезапное изъятие заменителя молока из рациона вело к изменениям обмена веществ. В переваривающей деятельности сложного желудка при отбивке могут наступить перебои, которые равным образом отражаются на обмене белков, углеводов, жиров и неорганических веществ. Свойственный для жвачных переворот в обмене веществ был бесперебойным, если диэтетический переход при отбивке не был причиной недостатка в питательных веществах и энергии. В противном случае характерный для взрослых жвачных низкий физиологический уровень сахара в крови развился медленно, содержание холестерина в крови повысилось, ибо организм ягненка недостаток энергии выравнил мобилизацией жиров.

В течение первых двух недель у ягнят возникла анемия, которая с началом нормального переваривания в рубце медленно исчезла. Если отбивка шла с перебоями, то анемия продолжалась дольше, была более тяжелой и отрицательно сказалась на развитии ягнят.

В искусственном выращивании ягнят бесперебойный способ поения, когда ягнята принимают корм согласно потребности, по авторам, лучше подражает физиологическим условиям, чем стадийный способ поения.

ЭКСПЕРИМЕНТЫ ПО РАННЕЙ ОТБИВКЕ ИСКУССТВЕННО ВЫРАЩЕННЫХ ЯГНЯТ. II. ОБМЕН АЗОТА И АМИНОКИСЛОТ У ИСКУССТВЕННО ВЫРАЩЕННЫХ ЯГНЯТ

МАРИЯННА СЕЛЕНИ-ГАЛАНТАИ, ЙОЛАН ЕЧАИ, Б. ЮХАС, Б. СЕГЕДИ и МАРИЯ ТЕЛЕКИ

Изучался обмен азота, метионина и лизина у искусственно выращенных ягнят в возрасте 2—42 дней, отбитых в 2-дневном возрасте после потребления молозива, содержавшихся индивидуально в клетках изучения обмена веществ. Эксперименты велись на трех группах животных. Эти группы отличались друг от друга в дозировке заменителя молока и времени отбивки. За время 38-дневного эксперимента 5 раз по 5 дней изучали обмен азота, метионина и лизина у ягнят. Между ретенцией азота и привесом, ретенцией азота и ретенцией метионина, дальше, между ретенцией азота и ретенцией лизина обнаружена умеренно тесная взаимосвязь ($r = 0{,}85$, $r = 0{,}82$ и $r = 0{,}79$ соответственно).

На основании данных исследований авторы пришли к заключению, согласно которому раннюю отбивку можно осуществить без перебоев в развитии таким образом, если молоко постепенно, в относительно длинный срок времени (в 12—32 дневном возрасте) изымут из рациона, ибо в рубце только в данном случае может развиться необходимая для переваривания грубого корма микрофлора и микрофауна.

ИЗМЕНЕНИЯ СОДЕРЖАНИЯ ПРОГЕСТЕРОНА И ТЕСТОСТЕРОНА В СЫВОРОТКЕ КУРЫ ОТ ДНЕВНОГО ВОЗРАСТА ДО ЯЙЦЕКЛАДКИ

Т. МУРАИ, ДЬ. ПЕТЕШ и П. ПЕЦЕЛИ

Радиологическим методом изучали изменения содержания прогестерона и тестостерона в периферийной сыворотке гибридных цыплят TR-90, в возрасте от одного дня по 3-х, 15-и и 22-недельный возраст. Концентрация прогестерона в сыворотке повышается по 15-недельный возраст, потом понижается непосредственно перед и после полового созревания и характерный для 15-недельного возраста уровень достигает к времени кладки первого яйца.

Уровень тестостерона в сыворотке не показывает изменений от дневного возраста вплоть до полового созревания. В возрасте от 15-и по 21-недельный возраст существуют 1 или 2 нерегулярных пика, которые к времени полового созревания становятся более регулярными и появляются в интервалах от 8 по 12 часов. Развитие овуляторного цикла ведет к переорганизации (новая синхронизация) суточных изменений уровня прогестерона и тестостерона в сыворотке.

ИЗМЕНЕНИЯ КОНЦЕНТРАЦИИ ПОЛОВЫХ СТЕРОИДОВ В СЫВОРОТКЕ У КУРЫ ВО ВРЕМЯ ЯЙЦЕКЛАДКИ

Р. ПЕЦЕЛИ, ДЬ. ПЕТЕШ З. СЕЛЕНИ и Т. МУРАИ

У кур породы хемпшпир 1,5-летнего возраста во время полового цикла изучалась концентрация прогестерона, эстрона и 17-β-эстрадиола в сыворотке. Радиоиммунологическое исследование проводилось после хроматографии на LH 20. Уровень прогестерона в сыворотке колебался в пределах 600 – 2500 пг/мл, показывая выраженный пик к 5 – 6 часам до овуляции. Уровень тестостерона в сыворотке колебался в пределах 25 – 320 пг/мл при отрицательной корреляции с прогестероном, показывая наивысшее значение 10 – 12 часов до яйцекладки, иногда превысив пик эстрогена. Колебания эстрона и 17-β-эстрадиола равнялись 40 – 330 и 25 – 720 пг/мл соответственно. Их наивысшие значения были неравномерными, некоторые — симультанными с пиками прогестерона. В регуляции овуляции прогестерон вероятно играет ведущую роль, но тестостерон и эстрогены согласно предположениям авторов проявляют регулирующее действие на метаболический процесс формирования яйца.

АБНОРМАЛЬНЫЕ ЭЛЕКТРОКАРДИОГРАММЫ У КРУПНОГО РОГАТОГО СКОТА, БОЛЬНОГО ЛЕЙКОЗОМ

З. ХОРВАТ и Т. СЕКЕРЕШ

Изучалась электрокардиограмма при сагиттальном отведении (электрода правой грудной конечности на шейной складке, левой грудной конечности — на холке, а левой задней конечности — на левой коленной складке) у 15 голов крупного рогатого скота с опухолями от лейкоза, у 12 голов в предопухолевом состоянии и с персистирующим лейкоцитозом и у 20 здоровых животных.

Получены удобные для сравнения электрокардиограммы. Наиболее заметные изменения электрокардиограммы лейкозного крупного рогатого скота являются следующие: а) фибрилляция, мерцание предсердий; б) удлинение электрической систолы, на что указывают увеличение кратного QT/TQ и PT/TP и повышение систолического индекса; в) удвоение комплекса QRS, повышение зубца R при II. и III. отведении; учащение изоэлектричности зубцов Q и S.

У одной части животных в предопухолевом состоянии эти ненормальности — в меньшей степени — тоже обнаружены.

Лейкозное заболевание сердца и формирование электрокардиограммы не всегда проходит параллельно. Согласно этому изучение электрокардиограммы ограниченной диагностической ценности.

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A NEW MICROBIOLOGICAL ASSAY SYSTEM FOR ANTIBACTERIAL DRUG RESIDUES IN ANIMAL ORGANS. ESTIMATION OF NIGERICIN RESIDUES

By

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(Received August 16, 1978)

Nigericin is a monensin-like antibiotic of polyether structure. In veterinary medicine it has been used as a coccidiostatic and anti-mycoplasmal drug. It has usually been by long-term feed medication.

The maximum permissible level of monensin in feeds has been established in the Food Additive Compendium (1975) as 90—110 mg/kg and a withdrawal from feeding period of 72 hr has been recommended to render the meat of the treated animals fit for human consumption. However the FAC presents no information about the maximum tolerable level of monensin residues in organs.

Residue assays after different periods of withdrawal, in organs from animals treated with therapeutic and excessive dietary levels of Nigericin are reported in this paper.

Detection of residual Nigericin was attempted in organs on the basis of the antibacterial action of the drug.

DONOHO and KLINE (1967) developed a thin-layer chromatography technique on silica gel. The spot of the separated antibiotic was detected on the thin-layer plates by the bioautographic method with *Bacillus subtilis*.

We found this procedure to be suitable for the estimation of relatively high levels ($\sim$ 125 mg/kg) of added Nigericin in feeds but not satisfactory to determine the very low levels of metabolically incorporated drug in animal organs.

The assay of antibiotic extracts from biological materials by the traditional agar-gel diffusion on punch plates presupposes the absence of any disturbing co-extracts, e.g. lipids. No satisfactory procedure has as yet been described for clean-up of Nigericin residues extracted from organs, and our previous efforts to developing one has failed. The contaminants disturb the diffusion in the agar gel and the irregularly-shaped, non circular inhibition zones are unsuitable for quantitative estimation.

A technique has been developed rendering measurements of the inhibition ring in agar plate unnecessary. After pervaporation of the lipid-like extract in a well of plexiglass and subsequent overlaying of inoculated agar medium described by DONOHO and KLINE (1967), in case of presence suitable amount of the active residue the microorganisms failed to proliferate on incubation, due to the inhibition, in and over the well.

This would give a "yes—non" answer.

To make this procedure apt for quantitation of the residues, a dilution principle was applied, namely series of twofold serial dilutions of the sample extract were placed in 10 wells.

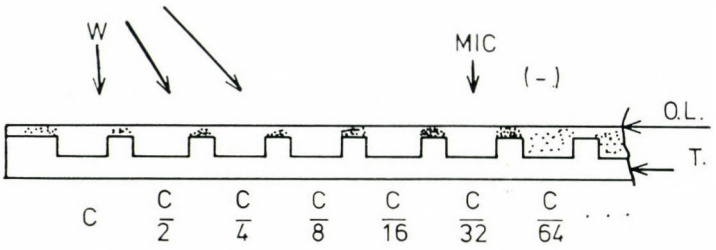

Fig. 1. Cross section of the assay tray; T, plexiglass assay tray; W, wells in the assay tray for twofold serial dilution of organ extracts and reference substance; c, concentration; O. L., overlay of the assay medium inoculated with *Bacillus subtilis* spores. The test organism fails to grow in and above the wells containing an inhibitory concentration of Nigericin; MIC, minimum inhibitory concentration; (—), the first dilution step representing a non-inhibitory effect of the antibiotic

In a neighbouring row of wells twofold serial dilutions of a known quantity of Nigericin were placed for reference. In the overlayed wells the inhibitory effect ranged several dilutions. An appropppriate adjustement of the quantitative relations made possible simultaneous estimation of the minimal inhibitory concentration (MIC) in both series in the same plediglass-tray covered with the same overlay. In the sample series the weight of the organ sample, in the reference series that of the pure active substance is known. The quantity of Nigericin present in the sample can be estimated as shown in Fig. 2. For technical details see below.

Materials and methods

Experimental animals

Young, healthy guinea fowls, turkeys, chickens and a lamb were used.

Treatment with Nigericin

a) Overdoses

Individuals of all four species were treated with excessive doses in aqueous suspension through a gastric tube. Part of the animals died in consequence of drug overdose; the survivors were killed after the treatment been concluded. Further informations in Table I.

The toxicological aspects were not pursued.

b) Therapeutic doses

Only broyler chickens were used in this trial. A diet containing 125 mg/kg Nigericin was fed to the birds for eight weeks. The chickens were kept in batteries. Individual variations were not pursued; pooled organs of five birds were assayed as one sample. Two groups of five animals were killed immediately after the conclusion of treatment, further groups in the 24th, 48th, 72nd and 96th hr of withdrawal, during which the same basal died was fed without Nigericin.

Further informations in table II.

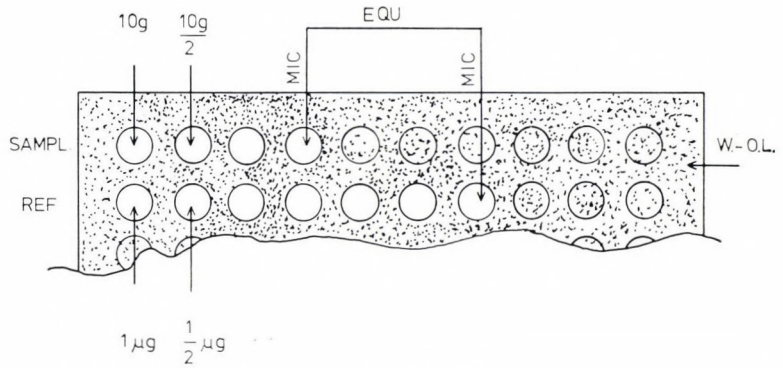

Fig. 2. Scheme of a quantitative evalution of residue levels in organ extract. W—O.L., wells in the assay tray and overlay of the assay medium; Ref., row containing the reference series; EQU, The arrows point to the wells containing the essentially equivalent minimal inhibitory concentration (MIC) of Nigericin in the reference and sample series. Evalution: the active substance content of the sample well filled with extract from 10/8 g sample corresponds to that of the reference well containing 1/64 µg Nigericin, i.e. ~0,01 mg/kg

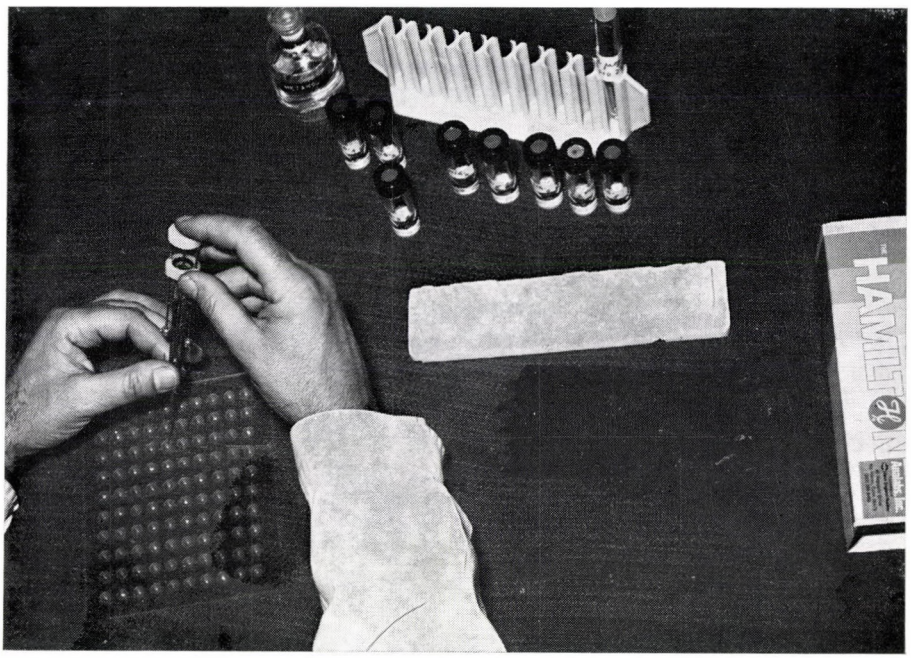

Fig. 3. Distribution of twofold serial dilutions in rows in the assay tray

c) Untreated controls

Two groups, each consisting of five birds, were set up as untreated control with the treated animals, under identical conditions in batteries, except for antibiotic supplementation. A single untreated bird (K/E in table II) had derived from another flock.

Table I

Residue levels on organs of animals treated with overdoses of Nigericin (The numerical data are intorpolations assessed by means of the reference grading described in the text

Species and designation of sample		Period of treatment/ number of medication days d : n	Individual total dose mg	Fate	Nigericin residue in liver in muscle mg/kg	
Guinea-fowl	Gf/1	1 : 1	160	died	1.6	1.6
	Gf/2	1 : 1	160	died	1.6	1.6
	Gf/3	1 : 1	160	died	1.6	1.6
	Gf/4	1 : 1	160	died	1.6	1.6
	Gf/5	1 : 1	160	died	0.8	0.4
Turkey	T/1	11 : 8	190	died	0.4	0.05
	T/2	15 : 11	310	killed	0.025	0.005
	T/3	15 : 11	310	killed	0.2	0.2
	T/4	15 : 11	310	killed	0.1	0.05
	T/5	15 : 11	310	killed	0.05	0.005
	T/6	15 : 11	310	killed	0.1	N. D.
	T/7	15 : 11	310	killed	0.4	0.05
	T/8	17 : 12	350	killed	0.4	0.4
	T/9	17 : 12	350	killed	0.2	0.05
	T/10	17 : 12	350	killed	Ø	0.025
	T/11	17 : 12	350	killed	0.05	0.025
	T/12	17 : 12	350	killed	0.1	0.05
	T/13	17 : 12	350	killed	0.4	0.05
Chicken	Ch/1	11 : 8	100	died	0.8	0.4
	Ch/2	1 : 1	120	died	0.8	0.1
	Ch/3	2 : 2	240	died	0.8	0.4
	Ch/4	12 : 9	120	died	0.1	0.05
	Ch/5	4 : 4	200	killed	0.4	0.2
	Ch/6	12 : 9	120	killed	0.005	0.005
	Ch/7	12 : 9	120	killed	0.005	0.005
	Ch/8	12 : 9	120	killed	0.025	0.0025
	Ch/9	12 : 9	120	killed	0.2	0.005
Lamb	L/1	16 : 13	1000	killed	0.025	0.025

Remark: Samples Gf/2, Gf/3 and Gf/4 were pooled, and the pool was divided into three parts which were further on tested separately

Ø, not tested; N. D., not detectable

Table II

Residue levels in the organs of broiler chickens treated with Nigericin at therapeutic dietary concentration (125 mg/kg)

Designation of organ pools from five birds	Period of treatment (weeks)	Drug withdrawal period	Residue levels in pooled samples of	
			liver	muscle
			mg/kg	
A/0	8	zero time	0.05	0.05
B/0	8	zero time	0.025	0.025
A/1	8	1 day	0.025	0.05
B/1	8	1 day (24 hr)	0.005	0.025
C/1	7	1 day	0.05	0.005
A/2	8	2 days	0.005	0.005
B/2	8	2 days (48 hr)	0.005	0.0025
C/2	7	2 days	0.001	N. D.
A/3	8	3 days (72 hr)	0.001	N. D.
B/3	7	3 days	N. D.	N. D.
A/4	8	4 days (96 hr)	N. D.	N. D.
B/4	7	4 days	N. D.	N. D.
K/I	untreated control group		N. D.	N. D.
K/II	untreated control group		N. D.	N. D.
K/E	untreated control bird		N. D.	N. D.

N. D., not detectable

Sampling and processing of samples

Individual or pooled specimens of organs, each weighing 20.0 ± 0.1 g, were minced and placed in 40 ml methanol. They were stored in tightly sealed vials in the refrigeator until used.

Extracts were prepared as proposed by DONOHO and KLINE (1967).

Preparation for assay

Preparation of the assay tray

Wells, 8 mm in diameter and 2 in the depth, were bored out in plexiglass plates of 20×20 cm, about 4 mm in thickness. A hundred wells were prepared per plate, spaced equidistant in 10 rows. The capacity of each well was slightly more than 50 μl.

Dilution and distribution of the sample extracts

The evaporated fat-like extract of each 20-g-sample was dissolved in ≤ 100 μl methanol and exactly its half amount (corresponding to 10 g sample) was placed with a microsyringe into the first well of a row. The remaining solution was then pipetted exactly half by half (each corresponding to 5 g

sample) into wells 2 and 3. To the content of well 3 then 25 μl methanol was added and after thorough mixing by pipetting the solution was accurately halved between wells 3 and 4 (each corresponding to 2.5 g sample). Then to the content of well 4 was 25 μl methanol added and the procedure was continued until the end of the row. Halving must not be effected by transfer of the calculad half volume, but always by exact halving the entire volume measured in the microsyringe.

To prepare the reference solution 1 μg nigericin is placed in the first well of the reference row, 0.5 μg in the second *etc.*

In a plexiglass tray two rows have been proved sufficient to reserve for the reference preparation; the remaining eight rows served for serial dilutions of organ extracts.

As the active substance of Nigericin remains stable, trays with completed reference and sample series can be stored until overlaying.

Conditions of the mocrobiological assay

Prepared assay trays were sterilized by a germicide lamp and in a carefully adjusted horizontal position overlayed as thinly and as possible with assay medium inoculated with *Bacillus subtilis* ATCC 6633 spores in a concentration as described by DONOHO and KLINE (1967). After solidification of the overlay, the trays were incubated for 16—18 hrs at 37° C.

During incubation the test organism formed colonies in the entire surface of the overlay except in and over the wells containing an inhibitory concentration of Nigericin.

The results must always be related to the reference series set up in the same tray.

Spore concentration in the medium is appropriate if the MIC in the reference series falls between wells 6 and 8.

The principle of evaluation is that the MIC of each sample and reference substance contains equivalent quantity of Nigericin with the accuracy of the 1 : 2 dilution step. Thus is easy to find the corresponding dilutions in the sample and reference series.

The results can be expressed as mg/kg and the accuracy correspond to gradation 1, $\frac{1}{2}$, $\frac{1}{4}$, *etc.*

Results

Assay of organ samples from untreated animals

Samples from 2 chicken muscles, 8 chicken livers, 2 turkey muscles, 2 turkey muscles, 2 turkey livers and 2 lamb livers, all from untreated animals

were investigated. In these "blanc samples" was no indication of growth inhibition. The reference series of Nigericin had its MIC in well 7 in two and in well 6 in the other two trays.

Inhibitory effect of Nigericin on addition to organ extracts from untreated animals

Extracts were prepared from muscle and liver of untreated chickens and turkeys, 4 individuum of each.

The entire extract of 20 g sample was placed in one wall (e.g. well 1 in a row) and the same with 0.025 μg Nigericin added in the well 2; in well 3 without organ extract, 0.025 μg Nigericin only was placed. Such assay tripletts were prepared of the 2 $\times$ 8 samples in two trays.

After incubation no bacterial growth inhibition was indicated in the wells containing the pure organ extract. Bacterial growth was equally inhibited in the presence and in the absence of organ extract. The present of the lipid extracts did not disturb the inhibition activity of Nigericin.

Recovery of Nigericin added

These trials were preformed at the 0.01 mg/kg range. 0.25 μg Nigericin, dissolved in 100 μl methanol, was added to each 20-g sample by means of a microsyringe; care taken to tistribute the antibiotic over as many organ fragments as possible.

Among the extracts tested in twofold dilution series in a total five trays, six chicken mucles samples and five chicken lever samples showed a shift to the left of the MIC by three wells.

The amount the first well of the reference series was 1 μg Nigericin and that of the sample series with the added Nigericin was $0.25/2 = 0.125$ μg. The dilution steps: 1 — 0.5 — 0.25 — 0.125 mg, the shift to the left corresponds to the amount added. For a single sample each of muscle and liver tissue the shift in MIC was four wells, i.e. more than expected. In another tray a single liver sample showed a shift of two wells. The extreme values, are 0.25 μg and 0.06 μg in the wells, i.e. 0.02 mg/kg and 0.005 mg/kg in the samples, instead of the added 0.0125 ($\sim$ 0.01) mg/kg.

Assay of organ samples from Nigericin-treated animals

The antibiotic residual levels in the organs found after treatment with overdoses are shown in Table I, along with data on the conditions of treatment and on the fate of the treated animals.

Data of residue assays in organ extracts chickens treated with Nigericin at the therapeutic dose level are presented in Table II, which also includes information about the main aspects of evalution.

Discussion

Assays for residues of Nigericin, a monensin-like drug were carried out by a new microbiological technique in organs from treated animals. The usual agar-gel diffusion technique on punch-plates was impracticable for microbiological assay, because we failed to eliminate the co-extracted lipids. In our technique the disturbing effect, arising from the diffusion of the co-extracts in the agar punch-plate has been eliminated.

Quantitative estimation of the residue levels was based on comparaison of a reference grading, which included eight approximative steps, i.e.: 0.1 — 0.05 — 0.025 — 0.01 — 0.005 — 0.0025 — 0.001 and $<$ 0.001 mg/kg. A matter of fact, these numerical data represent estimates rather than exact values, but make possible a good approximation within limits of error not exceeding one dilution step 1 : 2. This satisfies the required accuracy.

The reproducibility of the assay and a practically full recowery of test material were ascertainated that the presence of lipid co-extractes does not disturb the results under the given conditions.

Analysis of organs obtained from chickens treated for eight weeks with the therapeutic dose of Nigericin have shown that at this level of treatment, active residues appear in the organs at a very low concentration and become very rapidly eliminated, so that after a 72-hr withdrawal period prescribed for *monensin* in the Food Additive Compendium, practically no detectable residue of Nigericin was left. Thus, the sufficiency of the withdrawal period recommended on the basis of practical observations has ben substantiated experimetally by our new microbiological assay technique.

Detection of $<$ 0.001 mg/kg Nigericin can be regarded as a proof of the great sensitivy of the technique developed rather than of the inadequacy of the withdrawal period. Thus false negative results, which cause severe problems with many other assays, are not likely to occour at critical concentrations of residue in the present assay.

Summary

A new technique of microbiological assay has been developed for estimation of antibiotical drug residues in organ-extracts of Nigericin-treated animals.

The disturbing effect, arising from the diffusion of the co-extracts, in the usual agar punch-plate has been eliminated.

The punch-plates were replaced by plexiglass trays containing equidistal wells in 10 rows to hold appropriate serial dilutions of organ extracts and reference preparation. The liquid agar-medium, inoculated with test organism spores, was spread over the trays in the form of a thin overlay. During incubation the test organism formed colonies in the entire surface on the overlay except in and over the wells containing an inhibitory concentration of Nigericin. Quantitative estimation of the residue levels was based on the reference grading and it was possible to detect the residues even in the range 0.001 mg/kg.

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VACCINATION EXPERIMENTS AGAINST TRANSMISSIBLE GASTROENTERITIS (TGE) OF SWINE

VII. IMMUNOGLOBULIN CHARACTERISTICS OF ANTIBODIES IN MILK OF SOWS VACCINATED WITH THE "CKp" STRAIN OF TGE VIRUS

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Since CSONTOS and SZENT-IVÁNYI (1971) developed and orally applicable live-virus vaccine from the cell culture-attenuated CKp strain of transmissible gastroenteritis (TGE) virus in 1971, a great number of laboratory and field experiments have proved the innocuity and the immunogenicity of the vaccine and have outlined the mode of its administration (CSONTOS et al., 1973, 1974). The persistence of postvaccination titres and the antibody responses to revaccinations have been clarified in a series of field trials (BENYEDA et al., 1974). Further studies have elucidated the relationship between the virus titre and the antigenic effect of the vaccine (MOCSÁRI et al., 1976) and the question of the vaccine-virus excretion (MOCSÁRI, 1978). On the basis of the favourable experiences obtained during the field vaccination trials and TGE outbreaks, it has been suggested that orally vaccinated sows, as well as sows recovered from TGE, produce both IgA and IgG antibodies in the colostrum and milk and the passive immunity provided to their progeny is associated primarily with the level of the IgA antibodies in the milk.

In order to verify this hypothesis, we followed up the dynamics of specific TGE antibodies in the sera and milk wheys of sows that had been vaccinated orally or intramuscularly with the live CKp TGE virus, or had recovered from TGE. Thereafter, the immunoglobulin classes of lactogenic antibodies and their specific virus-neutralizing (VN) activity were determined.

Materials and methods

TGE vaccine. The live virus vaccine was prepared from the 44th passage of the CKp strain of TGE virus (CSONTOS and SZENT-IVÁNYI, 1971) on secondary swine thyroid cell cultures. The titre of the vaccine was $10^{5.25}$ $TCID_{50}$/0.1 ml. Its single dose, administered orally or intramuscularly (IM) was 5 ml.

Experimental animals. Thirty sows susceptible to TGE virus were orally and 10 were IM vaccinated twice at an interval of two weeks, seven weeks before the expected farrowing. Sows recovered from TGE four months before the collection of blood and milk samples served for comparison.

Collection and processing of blood and milk samples. Four sows with different VN antibody titres were selected from both the orally vaccinated and the naturally infected sow groups for representing the mean and also the

extreme titre values observed during the field experiments. Blood and milk samples were collected from them and from two IM vaccinated sows on the day of farrowing and throughout the 21-day lactation period. Blood samples were taken once a week, while milk samples every second day during the first week of lactation, and once a week thereafter.

The blood samples were taken from the vena cava superior. The milk samples were obtained following an intravenous injection of 25 to 30 IU oxytocin. A single milk sample represented the pooled milkings from two or three glands. Skimmed milk was prepared by the centrifugation of whole milk or colostrum at 2000 *g* for 30 min, then 1/10th volume of a 10% clotting substance was added and the samples were incubated at 37 °C for 60 min. After a second centrifugation at 2000 *g* for 30 min, the whey was collected by pipetting. Serum and whey samples were inactivated at 56 °C for 30 min, filtered through 0.45-μm membrane filters (MILLIPORE) and stored at —20 °C.

Specific TGE antibody titres of serum and milk specimens were determined by VN tests. Colostrum and milk whey specimens of three orally vaccinated sows, an intramuscularly vaccinated sow and one of the sows that had recovered from TGE were fractionated by Sephadex G-200 gel-filtration chromatography. Selected nonconcentrated fractions were tested for the presence of IgM, IgA and IgG by the double micro-immunodiffusion technique, and the TGE VN antibody titres were determined by the plaque-reduction test.

Virus strains. The CKp strain (CSONTOS and SZENT-IVÁNYI, 1971) and the FS 216/64 strain (CARTWRIGHT et al., 1965) of TGE virus were used in the VN test and in the plaque-reduction test, respectively. The latter virus strain formed smaller plaques at 37 °C than the CKp strain, a property advantageous for counting plaques.

Virus neutralization (VN) test. The materials and methods used were the same as described in previous papers of this series (BENYEDA et al., 1974; MOCSÁRI et al., 1975 a, b; MOCSÁRI et al., 1976; MOCSÁRI, 1978). The mean titre values were calculated from the logarithms of individual antibody titres.

Plaque-reduction test. The tests were carried out on secondary swine thyroid cells prepared in Petri dishes of 40 mm diameter by the method of THOMAS and DULAC (1976), slightly modified by us as follows: TGE virus was diluted to contain 50 to 80 PFU/0.1 ml dilution; the monolayers were overlaid with an agar medium that consisted of Parker's medium 199 (DIFCO) containing a final concentration of 5% calf serum and 0.8% Noble agar (DIFCO). Before counting plaques, the monolayers were fixed in a 10% solution of neutral formaldehyde, the agar overlays were removed and the monolayers were stained with an 0.015% solution of methyl violet as described by WITTE (1971). The antibody titre was expressed as the reciprocal of the sample dilution resulting a 50% plaque reduction. The formula of REED and MUENCH (1938) was used.

Gel filtration chromatography. Gel filtration was performed on Sephadex G-200 (PHARMACIA) in 0.1 *M* tris (hydroxymethyl)aminoethane — HCl buffer at pH 8.0 containing 0.2 *M* NaCl (TRIS/HCl). Following the procedure outlined by SAIF et al. (1972), two chromatographic columns (K25/45 and K 25/100, PHARMACIA) were connected in series, so that the flow was descendent in the first column and ascendent in the second. The total bed volume of gel was approx. 650 ml, the void volume (V_0) determined with Bue Dextran 2000 (PHARMACIA) was approx. 180 ml. Specimens were applied to the columns in volumes of 4.5 ml. The buffer system was TRIS/HCl. The flow rate was 8 to 10 ml/h accomplished by a UNIPAN 335 A type micro-pump.

Fractions of 3.5 ml were collected and their optical densities were determined at 280 nm (OD_{280}) in a SPEKTROMOM 204 (MOM) spectrophotometer. The 280 nm absorption profiles of samples were illustrated graphically.

Immunodiffusion. For double immunodiffusion, 1.5% Noble agar (DIFCO) was used in 0.146 *M* borate buffer at pH 8.6. A micro-modification of Ouchterlony's method was performed as described by WADSWORTH (1957). Rabbit antisera to porcine IgA, IgG and IgM were rendered monospecific for α, γ, and μ heavy chains by repeated absorptions by soluble antigens. Anti-IgA and anti-IgM sera were absorbed with small samples of porcine IgG [200 μg of IgG/ml of anti-IgA serum and 400 μg of IgG/ml of anti-IgM serum] while anti-IgG serum was absorbed with small samples of a mixture containing porcine IgA and IgM until no more anti-light-chain activity was detectable. The α_2-macroglobulin activity of antisera to porcine IgM was removed by absorption with precolostral piglet serum.

Results

Dynamics of specific TGE virus-neutralizing (VN) antibodies during lactation in the sera and milk wheys of sows that had been vaccinated orally or intramuscularly (IM) or had recovered from TGE

The dynamics of the mean antibody titres in the sera and milk wheys are shown in Fig. 1.

The individual serum antibody titres of orally vaccinated sows varied between 1 : 32 and 1 : 64 on the day of farrowing and remained constant throughout the lactation. The colostral antibody titres were between 1: 32 and 1 : 708 at farrowing, while those of milk wheys varied between 1 : 4 and 1 : 11 on the day of weaning (Fig. 1a).

The serum antibody titres of IM vaccinated sows were equally 1 : 256 on the day of farrowing and decreased by 2 $\log_2$ units during the lactation period. The colostral antibody titres were 1 : 512 and 1 : 1024 on the day of farrowing. However, titres in milk declined promptly after farrowing, so that

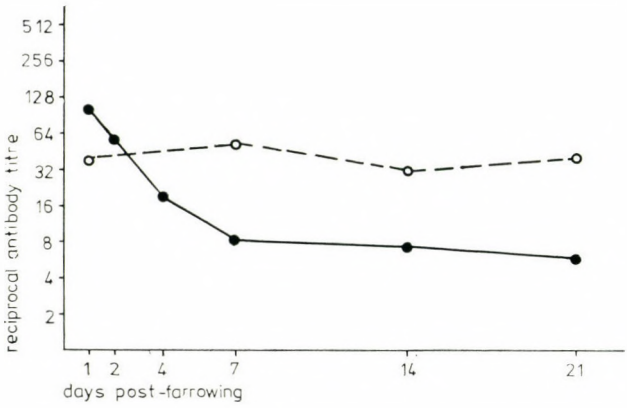


Fig. 1a. Dynamics of specific TGE VN antibodies in blood sera (– – –) and milk (——) from sows vaccinated orally

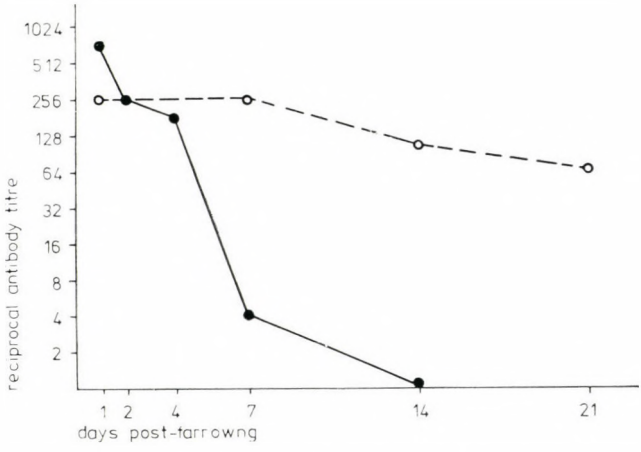


Fig. 1b. Dynamics of specific TGE VN antibodies in blood sera (– – –) and milk (——) from sows vaccinated intramuscularly

the samples taken 7 and 14 days post-farrowing (DPF) showed no TGE VN activity (Fig. 1b).

Blood sera collected from sows recovered from TGE neutralized 100 $TCID_{50}$ virus up to a dilution of 1 : 64 to 1 : 512 on the day of farrowing and these titres remained constant till the end of the lactation. The individual antibody titres of colostral wheys either equalled those of the corresponding serum sample or were higher than those by 1 $\log_2$ unit. At the end of the lactation period, the milk antibody titres varied between 1 : 11 and 1 : 32 (Fig. 1c).

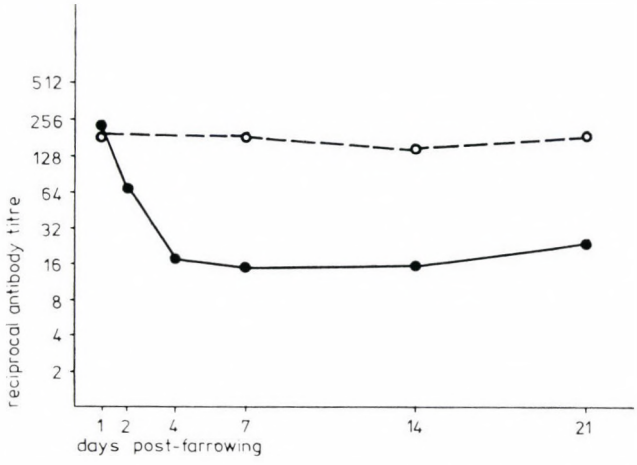


Fig. 1c. Dynamics of specific TGE VN antibodies in blood sera (– – –) and milk (——) recovered from TGE

Determination of the immunoglobulin (Ig) classes of lactogenic TGE viral antibodies and their VN activity

In the colostrum of orally vaccinated sows, IgG was the predominant Ig class. The concentration of IgA and IgM was several times lower. However, both the fractions containing IgG and IgA had high levels of TGE VN activity (Fig. 2a). By the 2nd to the 3rd DPF, the concentration of IgG decreased significantly in the milk, resulting in a several-fold VN titre reduction in this Ig region (Fig. 2b). From the 4th to the 8th DPF, the fractions of the IgG region had no TGE VN activity demonstrable by the plaque-reduction test. The concentration of IgA and the VN activity of the fractions containing IgA declined to a certain degree during the first three days of lactation but from the 4th DPF both remained practically constant until the end of the lactation (Figs 2c, 2d and 2e).

In colostral and milk samples of the IM vaccinated sow, the level of the three Ig classes and their ratios, were comparable throughout the lactation period to those found in the analogous chromatograms of the orally vaccinated sows' colostral and milk samples. IgG was the predominant Ig class in colostrum and the specific TGE antibody activity was associated primarily with this Ig class. The VN titres of the IgA and the IgM portions were several times lower than that of the IgG region (Fig. 3a). From the 2nd DPF, the VN activity of all three Ig classes declined parallel with their concentration. At weaning, only a very low concentration of IgG was found in the milk whey and, accordingly, the VN titre of the IgG fraction was extremely low (1 : 2.69). The IgA and the IgM fractions had no VN activity demonstrable by the plaque-reduction test (Fig. 3b).

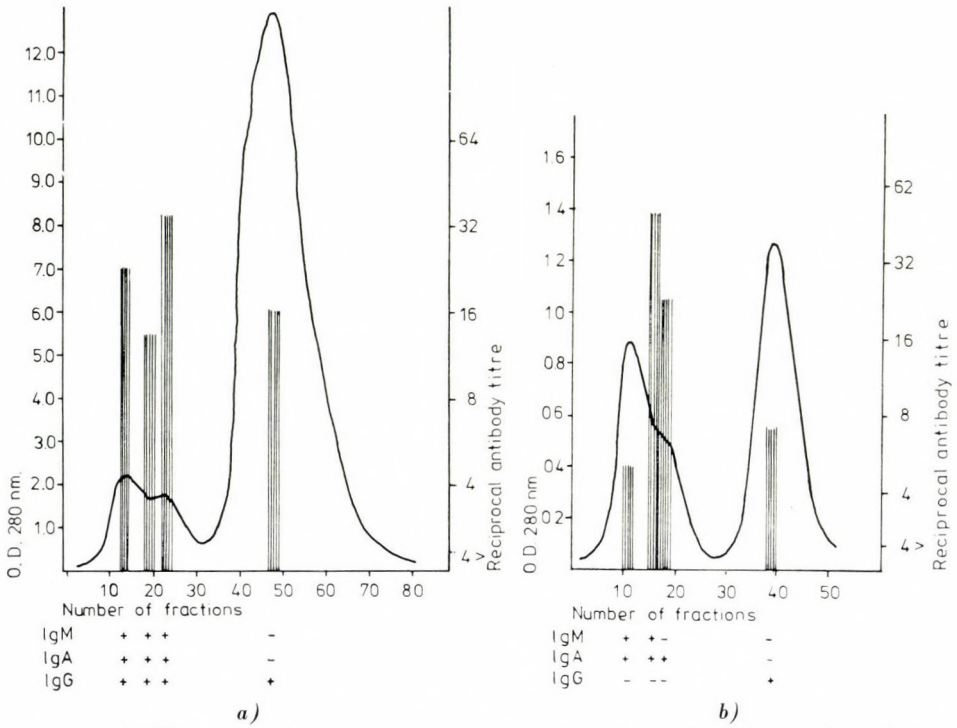


Fig. 2a—b. Gel filtration on Sephadex 200. Orally vaccinated sow No. 1359. *a*, colostral whey; *b*, 3-DPF milk whey. Explanation: vertical bars represent TGE VN antibody titres in selected unconcentrated fractions. Immunoglobulin classes were detected in these fractions by immunodiffusion using monospecific antisera to IgM, IgA and IgG. DPF, day postfarrowing

Similarly, IgG was the predominant Ig class in the colostral whey of the sow recovered from TGE as in those of orally and IM vaccinated sows. The specific TGE VN activity was also associated primarily with this Ig class but high VN titres were found in the IgA region as well (Fig. 4a). Starting from the 2nd DPF, the IgG concentration of the whey declined gradually, parallel with the VN titre of the IgG fraction. From the 14th DPF, the concentration of IgG was very low in the milk whey and, in accordance with this, no TGE VN activity could be demonstrated in the IgG fraction. On the other hand, both the concentration of IgA and the VN activity of the fractions containing IgA remained constant from the 4th DPF to the end of the lactation period (Figs 4b and 4c).

Discussion

The follow-up of the dynamics of specific TGE VN antibodies in the sera and milk wheys during lactation has enabled us to draw the following conclusions.

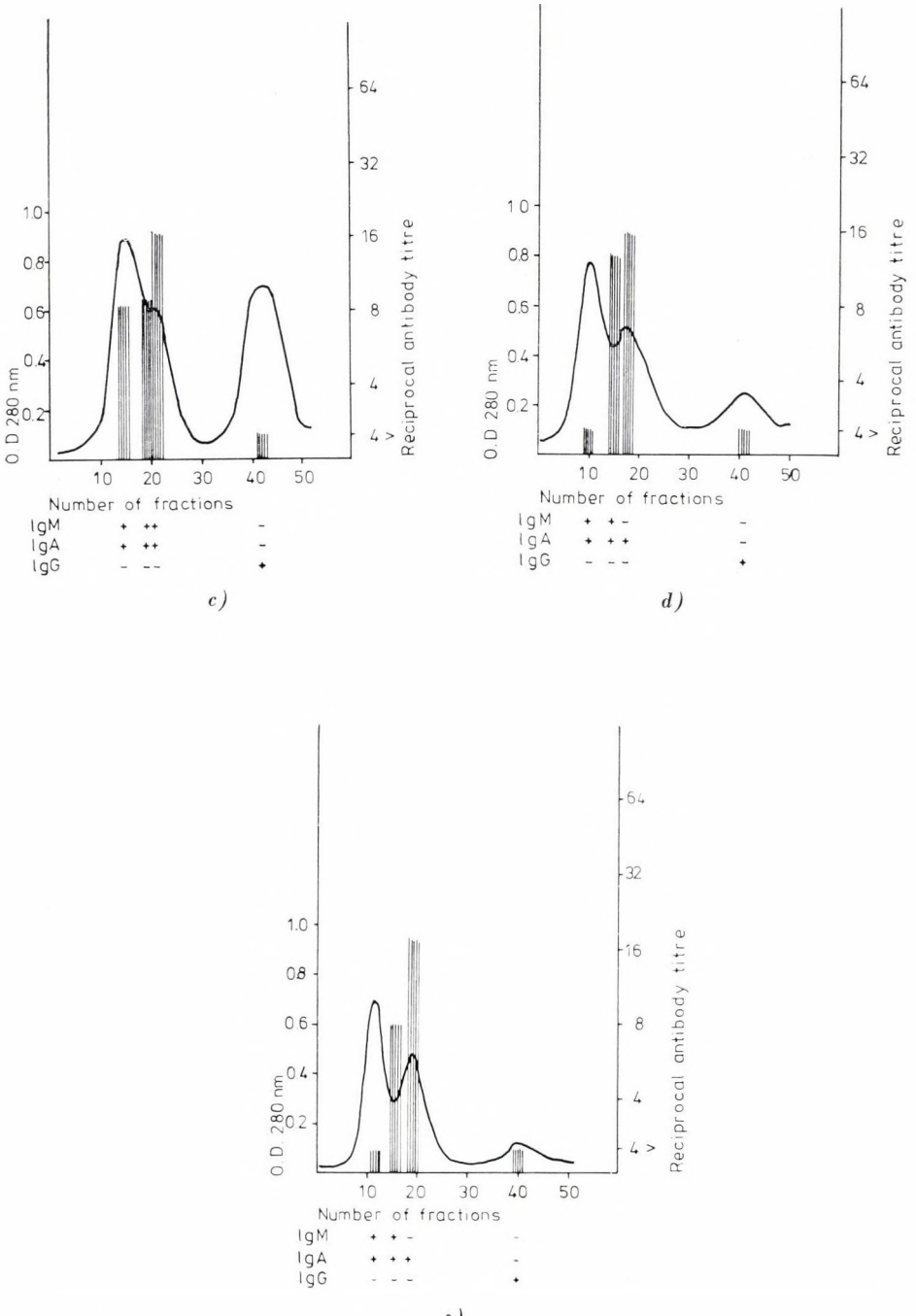


Fig. 2c—e. Gel filtration on Sephadex 200. Orally vaccinated sow No. 1359. *c*, 4-DPF milk whey; *d*, 13-DPF milk whey; *e*, 20—DPF milk whey. For explanations see Fig. 2a—b

The specific VN antibody titres were generally higher by 1 $\log_2$ unit in the colostral wheys of orally vaccinated sows than in their blood sera. From the 2nd DPF, the titres of milk wheys gradually declined. Between the 3rd to 7th DPF, their titres fell to a level 2 $\log_2$ units lower than the blood serum titre and then remained unchanged till the end of the lactation period.

The average VN antibody titre was higher by 0.5 $\log_2$ unit in the colostral wheys of sows recovered from TGE than in their blood sera. Similarly to the orally vaccinated sows, the titres of milk wheys decreased gradually and fell to a level of 2 to 3 $\log_2$ units lower than the mean titre of blood serum. Thereafter, they remained constant until the end of the lactation period.

Following IM vaccinations, the average VN antibody titre was higher in both the blood sera (8.0 $\log_2$) and colostral wheys (9.5 $\log_2$) on the day of farrowing than in those of orally vaccinated sows (5.3 and 6.7 $\log_2$, respectively) or of sows recovered from TGE (7.4 and 7.8 $\log_2$, respectively). Subsequently, the titre of milk wheys declined markedly and between the 7th to 14th DPF, the milk whey samples had no demonstrable TGE VN activity. The mean antibody titre of blood sera showed a similar decreasing tendency. Their average titre was lower by 2 $\log_2$ units at the end of the lactation than at the farrowing time.

Similar findings were reported by BOHL et al. (1972, 1975). They found likewise considerably higher antibody levels in the blood sera and colostral wheys of IM or intramammarily vaccinated sows than in those of sows infected orally with virulent TGE virus. The same authors reported marked declines of VN antibody titres in the parenterally vaccinated sows' milk (BOHL et al., 1975). In an IM vaccinated sow, *e.g.*, the VN antibody titres were 1 : 1350 in the colostrum, 1 : 70 in the 4-DPF milk and 1 : 6 in the 7-DPF milk, as were determined by plaque-reduction tests.

Concerning the concentration of the Ig classes and their ratios, significant differences could not be demonstrated among the gel filtration chromatograms of analogous colostrum and milk samples from sows that had recovered from TGE or had been vaccinated against TGE by different routes. IgG was the predominant Ig class in all of colostral wheys. The concentration of IgG decreased gradually from the 1st DPF and rapidly from the 3rd or 4th DPF. Starting from the 14th DPF, very low concentrations of IgG were found in the milk. During the first few days of lactation, the concentration of IgM fell to half the level found in the colostrum, but it showed only a moderate decline from the 4th DPF until the end of the lactation period. Similarly, the concentration of IgA fell to approx. half the level of the colostral IgA concentration within the first 48 hr postpartum. Thereafter, its level remained practically constant until the end of the lactation period and so, owing to the rapid decline of IgG concentration and the gradual decrease of IgM concentration, approx. from the 14th DPF, IgA became the predominant Ig class in milk.

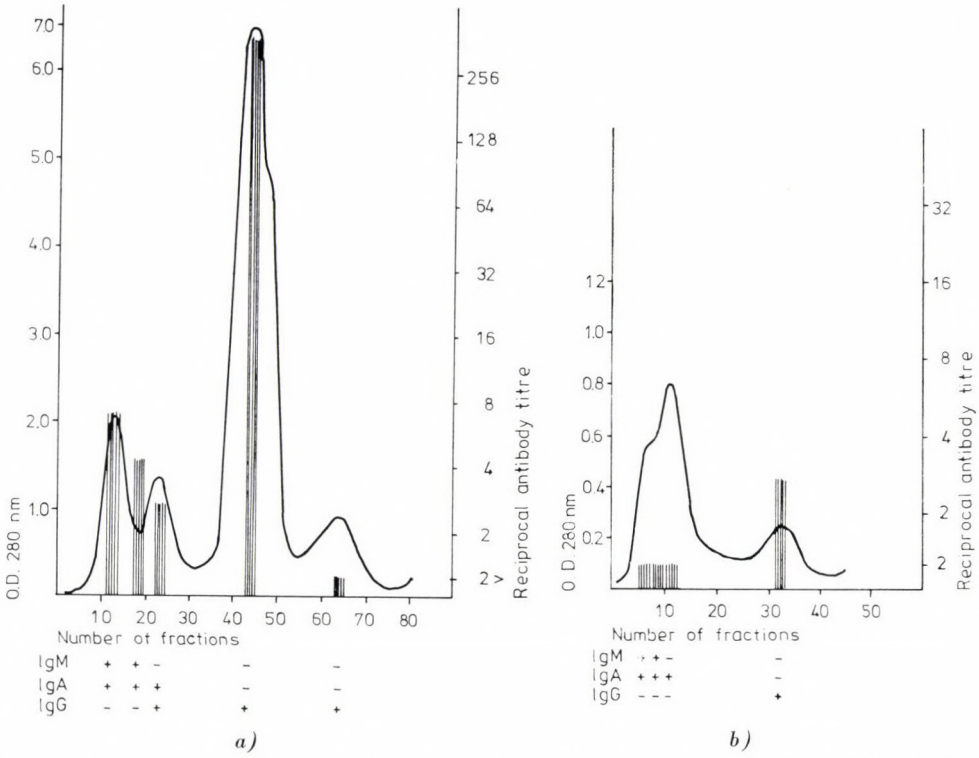
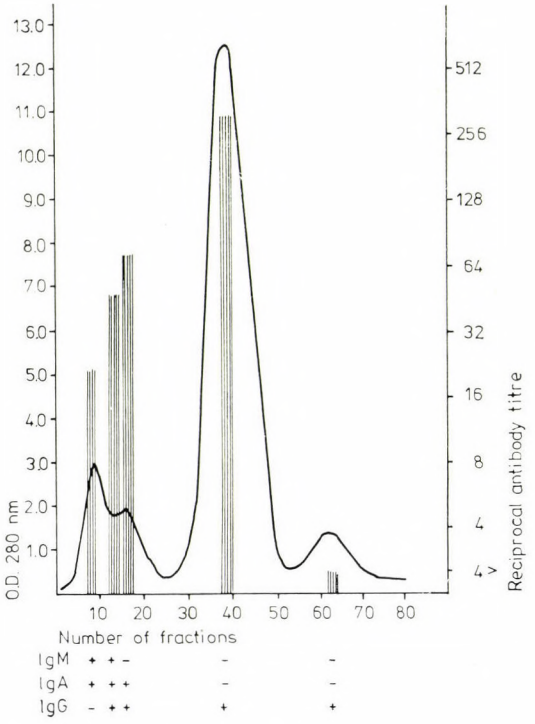


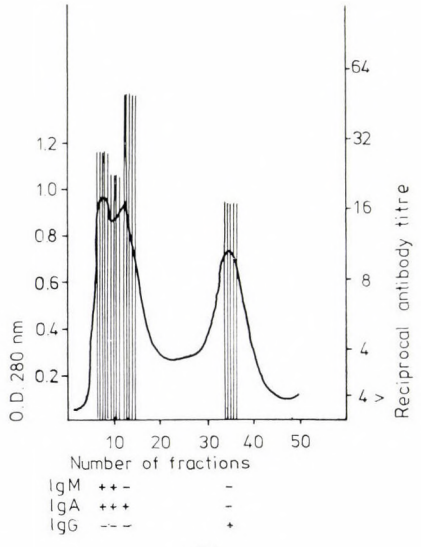
Fig. 3a—b. Gel filtration on Sephadex G-200. Intramuscularly vaccinated sow No. 968. *a*, colostral whey; *b*, 22-DPF milk whey. For explanations see Fig. 2a—b

Concerning the VN activity of different Ig classes, significant differences were observed between sows that had recovered from TGE or had been vaccinated orally and the IM vaccinated sow. It is a well-established fact that in colostrum and milk of sows that have recovered from TGE or infected orally with virulent TGE virus, IgA is the predominant Ig class of TGE antibodies. On the other hand, TGE antibodies in colostrum and milk are primarily or solely of the IgG class in parenterally (IM, intramammarily or subcutaneously) inoculated sows (Abou-Youssef and Ristic, 1971 1972, 1975; Bohl et al., 1972 a, b; Ristic and Abou-Youssef, 1972; Saif et al., 1972; Stone et al., 1974). Similar results were obtained in the present study analysing the Ig characteristics of antibodies in colostrum and milk from naturally infected and IM vaccinated sows.

In orally vaccinated sows, similarly to the sows recovered from natural infection, the specific VN activity of lactogenic TGE antibodies was associated primarily with IgA. Although high levels of IgG antibodies occurred in the colostrum, the concentration and, consequently, the VN activity of this Ig



a)



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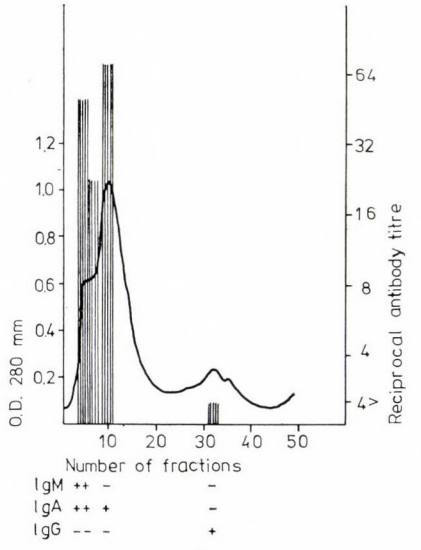

c)

Fig. 4a—c. Gel filtration on Sephadex G-200. Naturally infected sow No. 5. *a*, colostral whey; *b*, 4-dPF milk whey; *c*, 21-DPF milk whey. For explanations see Fig. 2a—b

class decreased rapidly in the milk. From the 4th to the 6th DPF, IgG had no TGE VN activity demonstrable by the plaque -reduction tests. The concentration of IgA and the titre of TGE antibodies of the IgA class, as in an immunological response after natural infection, decraesed to a certain degree during the first three days post-farrowing, but thereafter both remained constant until the end of the lactation period.

The results obtained have given a clear explanation of the results of studies following up the dynamics of lactogenic TGE antibodies during lactation. Antibody titres in milk of all orally vaccinated or naturally infected sows declined by 2.5 to 3.5 $\log_2$ units during the first few days post-farrowing. This comes principally from the marked fall of IgG concentration observed between the 2nd to 7th DPF and, consequently, from the decreasing VN activity of this Ig class. The VN activity persisting practically at a constant level from that time until the end of lactation is primarily, if not solely, associated with the specific TGE VN activity of the secretory IgA, the concentration of which remains constant from the 4th DPF until the end of lactation and it constitutes the predominant class of Ig in milk after the first week of lactation.

In the IM vaccinated sow, as a result of parenteral antigenic stimulation, TGE antibodies in colostrum and milk appeared to be primarily, if not solely, of the IgG class. Consequently, antibody levels in the milk fall rapidly, parallel with the concentration of IgG. Depending on the initial level of TGE VN activity, from the 7th to 14th DPF, when the concentration of IgG falls under a certain level, the specific VN activity of the milk also disappears.

The results of this study have been at variance with the establishments of Bohl and Saif (1975). These authors vaccinated pregnant sows with the live Purdue strain of TGE virus that had been serially passaged 114 times in porcine kidney cell cultures (P-114). After oral(intranasal) vaccinations, TGE antibodies in milk were primarily of the IgG class, besides low levels of IgA antibodies were detected in three of six vaccinees. One of the reasons of this controversy might be the variance in the immunogenic properties of the P-114 and CKp strains of TGE virus. This may be one of the original properties of the Purdue strain or it may have developed during the 114 cell culture passages. The results of vaccinations with the B1 strain of TGE virus reported by Bachmann and Hess (1978) seems to support the latter assumption. In sows that had been vaccinated twice with the B1 TGE virus given orally in entero solvent capsules, the 300 times passaged attenuated virus induced a higher IgA- than IgG antibody titre in colostrum and 7-DPF milk. On the contrary, the 350 times passaged B1 virus, as the P-114 strain of TGE virus, stimulated primarily TGE antibodies of the IgG class.

Summary

The dynamics of specific TGE VN antibodies were followed up during lactation in the sera and milk wheys of sows vaccinated orally or IM with the CKp strain of TGE virus and of sows recovered from TGE. Further, the Ig classes of lactogenic TGE antibodies and their specific VN activity were determined.

The average VN antibody titre was higher by 1 $\log_2$ unit in the colostral wheys of orally vaccinated or naturally infected sows than in their serum samples taken at the time of farrowing. Between days 3 and 7 post-farrowing, the VN titre of milk wheys fell to a level of 2 to 3 $\log_2$ units lower than that of blood sera. Thereafter, the titres remained practically constant up to the end of lactation. Following IM vaccinations, the mean VN antibody titre was higher in both the blood sera and colostral wheys than in those of orally vaccinated or naturally infected sows. However, from the first day of lactation, the antibody titres declined markedly in milk of IM vaccinated sows and they disappeared between days 7 and 14 post-farrowing.

In orally vaccinated sows, the specific VN activity of lactogenic TGE antibodies was associated primarily with IgA just as in sows recovered from TGE. Both in orally vaccinated and naturally infected sows' milk, the titre declines of 2 to 3 $\log_2$ units, compered to the serum titre, originate meanly from the marked fall in IgG concentration between days 2 and 7 post-farrowing and, consequently, from the decreasing VN activity of this Ig class. The VN activity persisting practically at a constant level from that time on until the end of lactation is primarily, if not solely, associated with the specific TGE VN activity of secretory IgA, the concentration of which remains constant between the 4th day post-farrowing and the end of the lactation period.

In the IM vaccinated sows, as a result of parenteral antigenic stimulation, lactogenic TGE antibodies appeared to be primarily, if not solely, of the IgG class. Consequently, antibody levels in milk fall rapidly, parallel with the concentration decline of IgG. Depending on its initial level, the specific VN activity of milk disappears between days 7 and 14 post-farrowing, *i.e.* at the time when the IgG concentration in the milk whey falls under a certain level.

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REACTION OF DOGS TREATED WITH IMMUNOSUPPRESSIVE AGENTS TO INFECTION WITH THE VIRUS OF RUBARTH DISEASE

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The causative agent of the viral hepatitis of dogs (VHD) (Rubarth disease) is an adenovirus. Serological studies performed in various countries have shown that a high percentage (20—70%) of dogs undergo an infection with the virus, often without apparent clinical symptoms. Occasionally, however, the disease is very severe and animals may die 1—6 days following infection. In such latter peracute or acute cases, haemorrhagic liver dystrophy develops.

According to MIMS (1964) macrophages may have different roles during the course of virus infection. In some viral diseases, macrophages of the liver take up virions, which then replicate in them.

Works dealing with the pathogenesis of viral hepatitis in dogs (RUBARTH, 1947; INNES, 1949; LINDBLAD and BJÖRKMAN, 1964) have pointed to the impairment of the sinusoidal walls in the liver. COFFIN et al. (1953) and later GIVAN and JÉZEQUEL (1969) reported on the greater frequency of viral inclusion bodies in the sinusoidal Kupffer cells in artificially infected dogs. Our recent liver biopsy studies on dogs with VHD (KAPP, 1977) have shown that the pathogenic adenovirus first affects Kupffer cells, and the hepatic injury developing in the course of the disease does not start with hepatosis in the strictest sense of the word, but with the impairment of the mesenchymal part of the liver (capillarization), and that the adenovirus responsible for VHD is primarily endotheliotropic.

Since the adenovirus causing VHD mainly affects the cells of the immune system in the liver and replicates in these cells, it was assumed that the state of RHS cells (either free of blocked) might be basically involved both in the mild and the severe form of the disease. The blocked state of RHS cells may favour the development of peracute or acute lethal VHD (acute liver dystrophy) To verify this hypothesis, we treated one part of our experimental dogs with an immunosuppressive agent at the time of the virus infection.

Materials and methods

Four, four-month-old German sheep-dogs from the same litter were used in the experiment (no. I: male, 6750 g; no. II: male, 7000 g; no. III: female, 6200 g; no. IV: female, 5000 g; healthy dogs).

The body temperatures of dogs varied between 38.3—39.0 °C. Erythrocyte count was 5.7—6.5 million/μl, leucocyte count between 8000 and 11 000/μl. The percentual ratio of lymphocytes and granulocytes varied between 24—26% and 61—72% respectively.

Mode of infection. The four dogs were infected with the liver homogenate of a dog which had fallen ill with VHD spontaneously and died. Each dog was given i.m. 0.4 ml of liver homogenate diluted about tenfold in saline, i.m. Simultaneously with the virus infection two dogs: no. II and no. IV. received a cytostatic drug impairing the cellular immune system (Lycurim R, Gedeon Richter Pharmaceutical Works, Budapest, Hungary). Dogs no. II and IV were given a single dose Lycurim i.v., 105 mg and 75 mg, respectively.

In the diseased dogs body temperature and blood picture were followed up and in the needle biopsy specimens were examined on the 2nd and 3rd day following infection.

Results

In the dogs which were infected with the virus of VHD, and were partly treated, partly not treated with Lycurim, the temperature increased from the first day of infection and then persisted between 40 and 40.6 °C (Fig. 1). In the period of fever, the animals were faint and had no appetite.

In dogs no. II and no. IV, which were treated with the virus of VHD and received Lycurim simultaneously, there was a significant decrease in leucocytes on the 2nd day. Leucocyte count was 3900/μl and 2300/μl in dogs no. II and no. IV. Of the leucocytes, the lymphocytes decreased considerably. In dog no. II their ratio was 5% and in dog no. IV 1%.

On the 3rd day following infection blood cells counts were similarly low in dogs no. II and no. IV, *viz.* leucocyte count was 2100—2800/μl, and the ratio of lymphocytes 1—3%.At this point of time erythrocyte count was also decreased: 3.8 million/μl and 4.1 million/μl in dogs no. II and no. IV, respectively.

In the liver biopsy specimens of dogs no. II and no. IV simultaneously given virus and immunosuppressive drug, alterations were observed electronmicroscopically in the Kupffer cells of liver sinusoids during the disease. On the 2nd day following infection, the dilatation of liver sinusoids was common and considerably enlarged and deformed Kupffer cells were sporadically in the sinusoidal walls. On the surface of enlarged and irregular Kupffer cells, deep invaginations and numerous, long and widened pseudopodia developed. In the Kupffer cells, the electron density of the cytoplasmic matrix became more pronounced and among the more or less preserved cell organelles the number of phagolysosomes increased. The nucleus was marginal, in general, and the nucleoplasm consisted of coarse granules of high electron density (Fig. 2).

With the progression of the disease (on the 3rd day following infection), in a great proportion of sinusoids phagocytized thrombocytes and leucocytes and their remnants were found in the cytoplasm of activated Kupffer cells (Figs 3 and 4). At the same time, signs of severe injuries were visible in the majority of Kupffer cells in the sinusoids of the liver. In many Kupffer cells possessing large pseudopodia, the cytoplasm became homogeneously electron

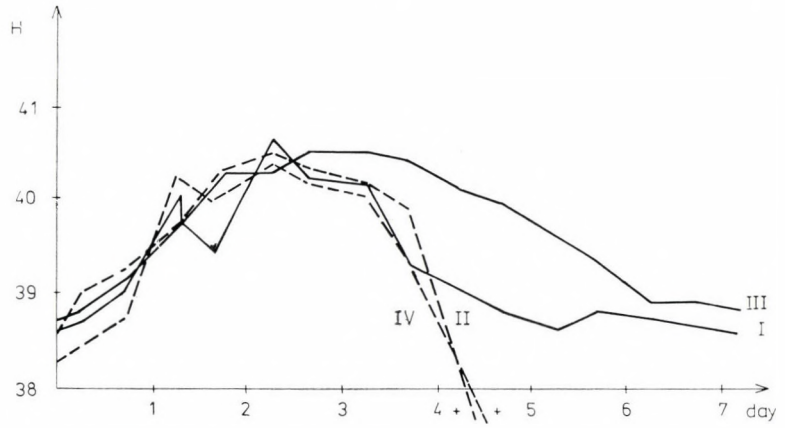

Fig 1. Reaction of dogs infected with the pathogen of viral hepatitis and simultaneously treated with Lycurim. Dogs no. II and no. IV were given virus im. + Lycurim iv. Dogs no. I and no. III were given virus im. only

dense, and the cytoplasmic organelles were difficult to recognize, or were completely destroyed. In the nucleus, the nucleolus was considerably enlarged, in general, its matrix was homogeneous and definitely electron dense (Fig. 5). In the liver biopsy specimens, Kupffer cells containing virus particles could also be observed occasionally. The nuclei of these were significantly enlarged, and there were concentrations of chromatin along the destroyed nuclear membrane. A few virus particles were observable in the nuclear matrix, in the damaged cytoplasm and free-standing in the sinusoidal hole (Fig. 6). Among the sinusoidal endothelial cells, elongated red blood cells which extended in the direction of the space of Disse were often visible.

In the sinusoidal wall, the endothelial cells were slightly loosened, in some places the endoplasmic reticulum was slightly dilated in the cytoplasm. In several places, the space of Disse was filled with a fine granular, moderatley electron dense substance. The microvilli of hepatocytes were occasionally more expressed than normally. The cytoplasmic organelles of hepatocytes showed no particular changes. Glycogen granules occurred in moderate or great amounts in the cytoplasm of parenchymal cells. Virus replication could not be observed in hepatocytes.

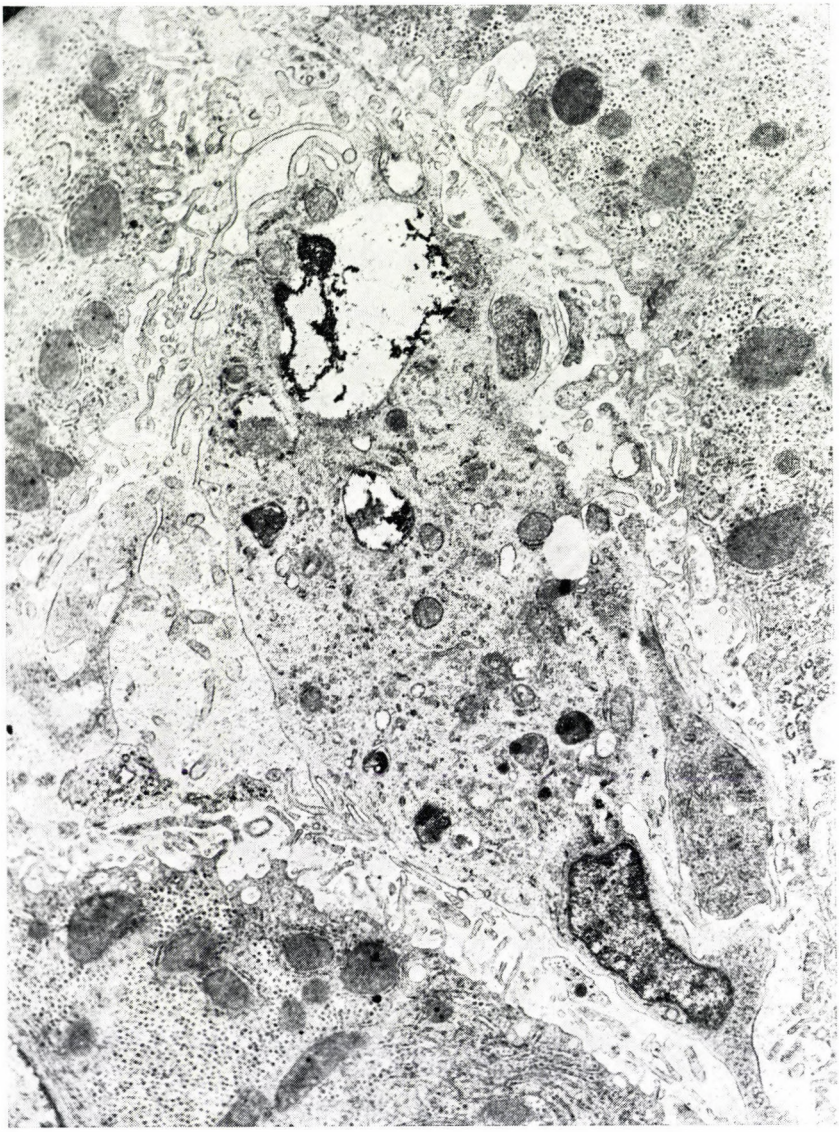

Fig. 2. Enlarged Kupffer cell with deep invaginations and long microvilli. ×9700

Blood samples of control dogs no. I and no. III infected with the virus VHD and not treated with immunosuppressive agent, showed only a slight decrease of leucocyte count and erythrocyte count on the 4th day of the disease. Between the two dogs there was no difference in the quantitative blood picture. Among the leucocytes, the ratio of lymphocytes was 23% and 28%

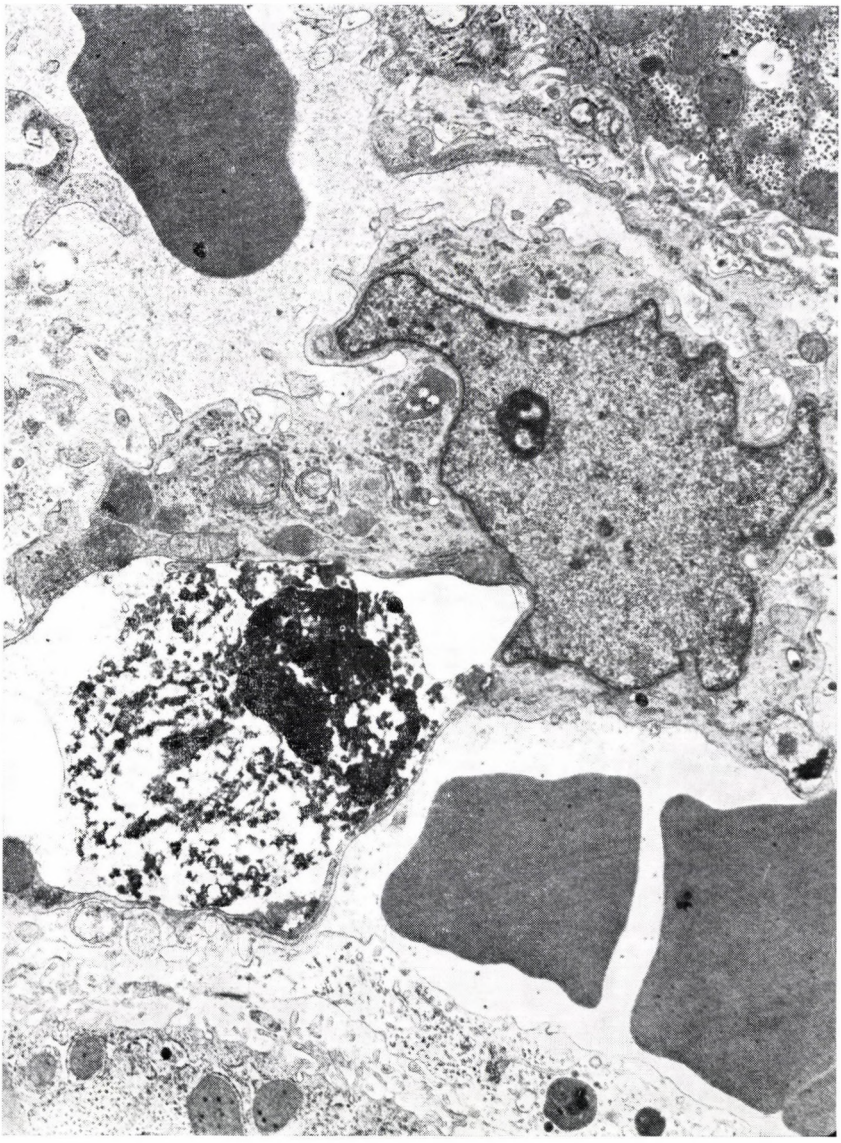

Fig. 3. Dead cell remains in the activated Kupffer cell. ×9700

in dogs no. I and no. III. respectively. Liver biopsy specimens from these two animals showed the sinusoidal walls generally preserved during the disease (on days 2 and 3 following infection). In some sinusoids, sporadical necrosis of Kupffer cells was observed, and signs of virus replication were occasionally visible in the nuclei of Kupffer cells. Glycogen content was generally rather

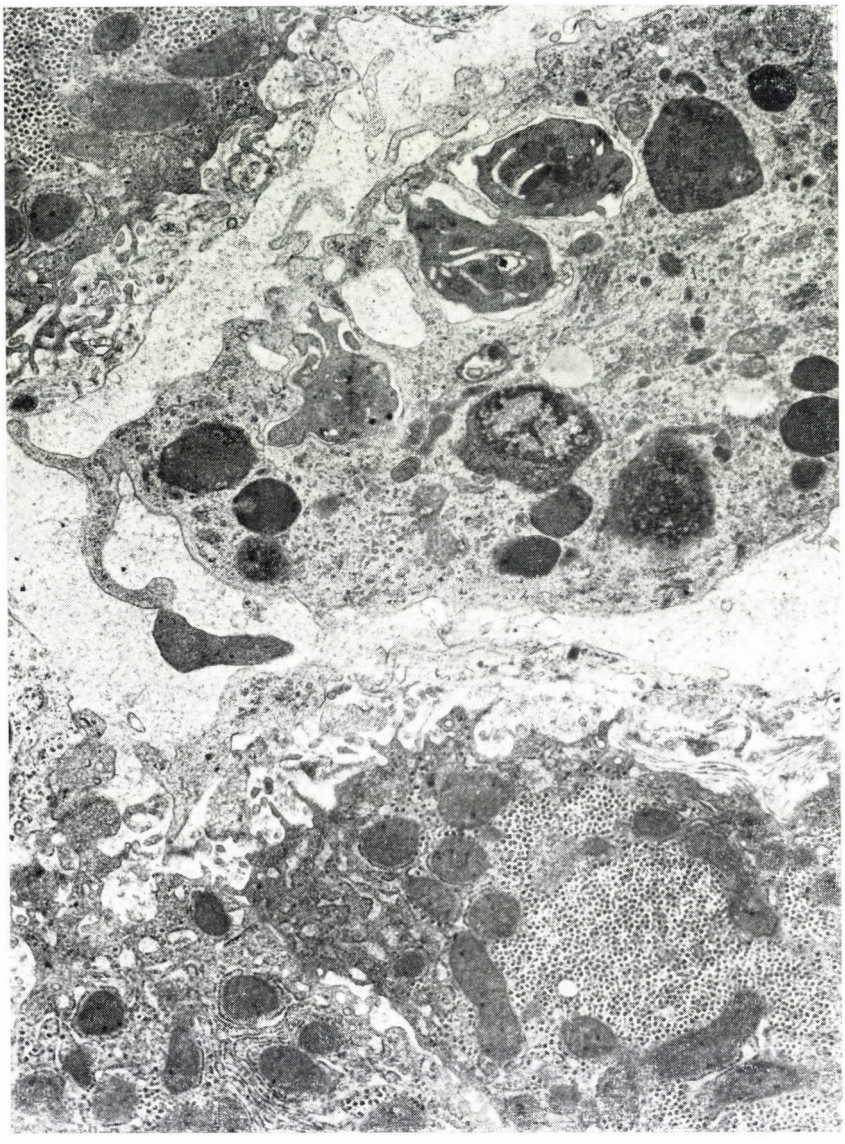

Fig. 4. Phagocytized thrombocytes and leucocytes in a Kuppfer cell possessing pseudopodium. ×11 100

high in the parenchymal cells of the liver, and in them virus replication or other injuries were not observable.

Both infected being under immunosuppressive effect (dogs no. II and no. IV) died on the 5th day after infection. Histologically acute haemorrhagic dystrophy was observed in the livers of both. There were Cowdry A-type nuclear inclusions in a great proportion of the parenchymal cells (Fig. 8b).

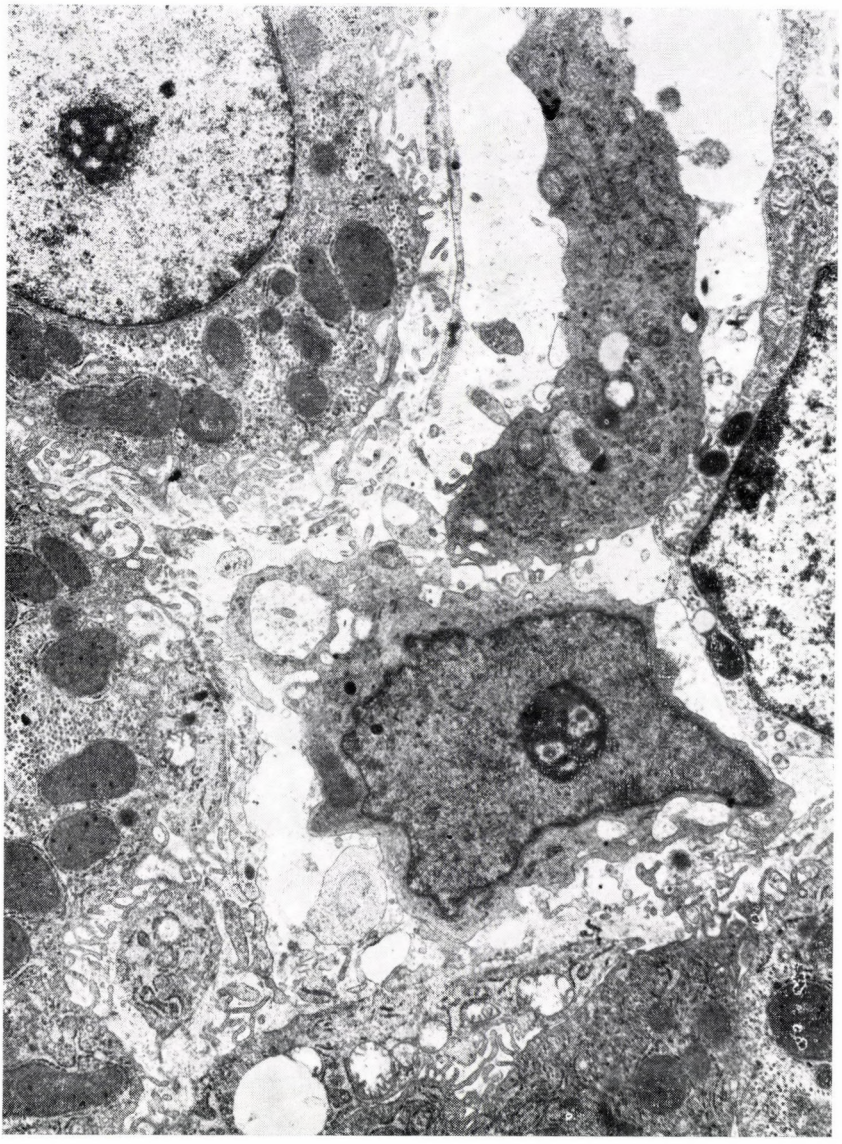

Fig. 5. Injured Kupffer cells in liver sinusoid. ×9700

In electron micrograms extensive virus replication was seen in the area of nuclear inclusions, in the hepatocytes (Fig. 8). Concerning the other organs of the dead animals, extensive haemorrhage was found in the mucous membrane of the stomach and intestines, and in the mediastinum there was edematous infiltration. Spleen and lymph nodes were reduced in size and their lymphoid elements were decreased.

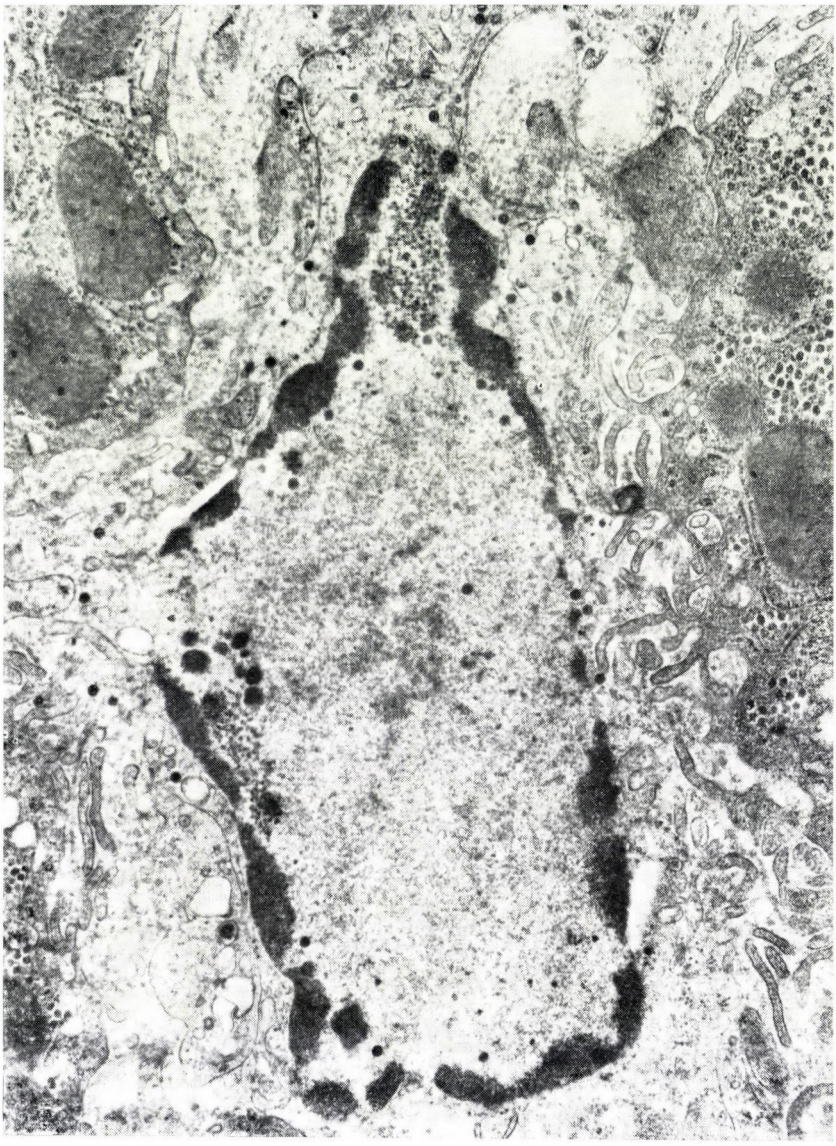

Fig. 6. Virus replication in Kupffer cell and free virus particles in the space of Disse. ×20 800

Dogs, no. I and no. III, which were infected with the virus of VHD and were not treated with immunosuprressive drug, survived. On the 5th-6th day following infection their temperatures became normal and they seemed clinically healthy. On the 14th day of infection, the sera of these surviving dogs contained antibody against the virus of VHD even in a high dilution.

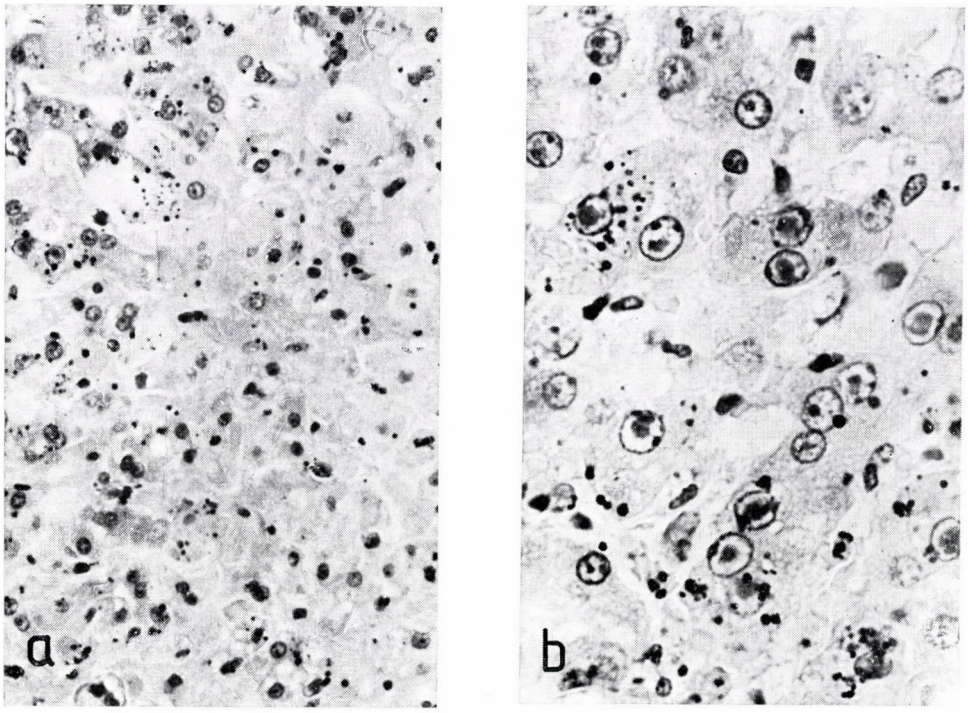

Fig. 7a—b. a, Acute liver dystrophy. H and E. ×400 *b*, Cowdry A-type nuclear inclusions in a great proportion of hepatocytes. H and E. ×1000

Discussion

Four susceptible dogs from the same litter were infected in the same way with lethal doses of an organ homogenate containing the virus of VHD. Out of the infected dogs, those two which had also been given an immunosuppressive drug (Lycurim cytostatic) died of hepatitis within a few days, the other two survived.

In the two dogs treated with both virus and immunosuppressive agent the significant decrease in lymphocytes in the lymphoid organs and the blood may be ascribed to the effect of the cytostatic drug. According to the examinations, first of all the number of lymphocytes is reduced by a small dose of the drug.

In liver biopsy specimens taken from dogs artificially infected and simultaneously treated with Lycurim, the Kupffer cells were considerably hypertrophic and numerous microvilli and pseudopodia were formed on the sinusoidal surface. Changes of this kind and dimension were not observed in control animals not treated with Lycurim.

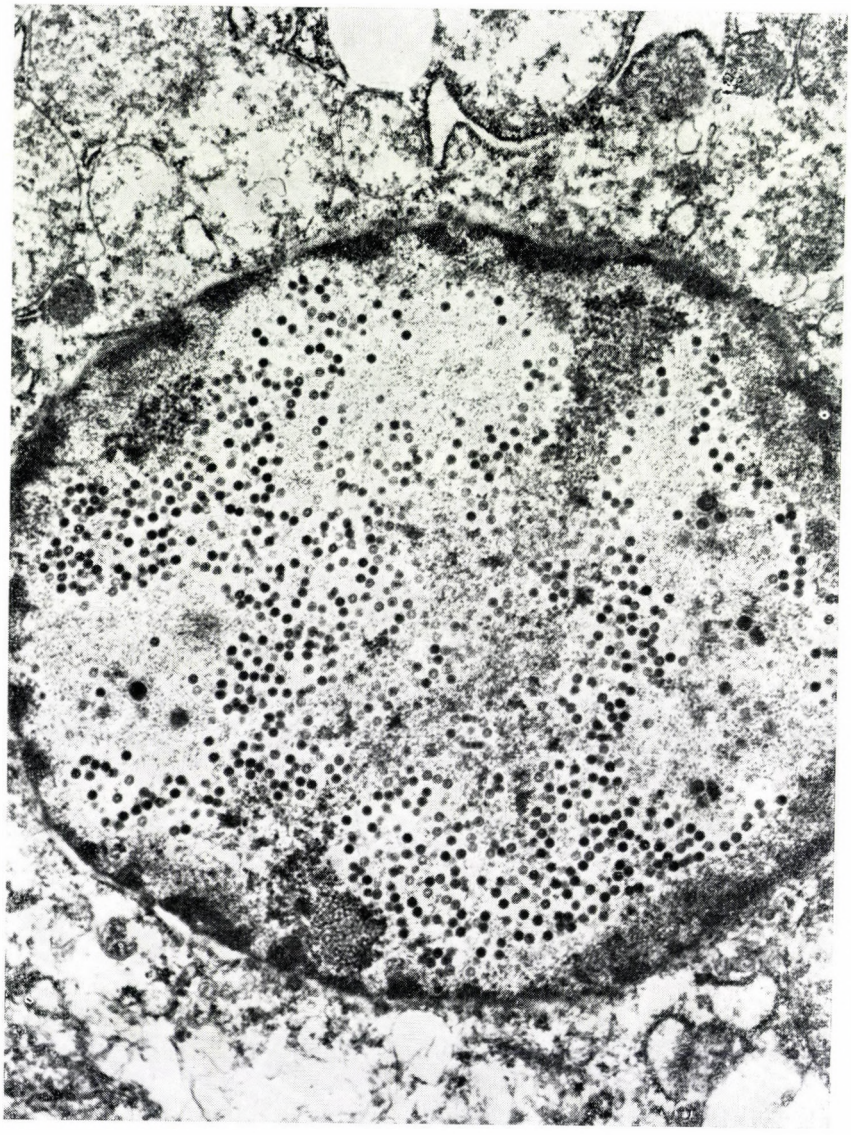

Fig. 8. Marked virus replication in the nucleus of a hepatocyte. × 20 800

Phenomena similar to the changes in Kupffer cells described in the foregoing were observed by FAITH and TRUMP (1966) in humans with acute glomerulonephritis, in the capillaries of the glomeruli. The changes manifested themselves in the hypertrophy of the endothelial cells. BECKER and GATZKE (1972) observed similar alterations in the endothelial cells of small blood vessels of rabbits and pigs following infection with *Herpesvirus suis*. The hyper-

trophy of the endothelial cells associated with pseudopodium formation was believed by these authors to be a nonspecific adaptation phenomenon which occurs in the endothelial cells of during various diseases. The altered behaviour of hepato-sinusoidal macrophages in the diseased dogs treated with Lycurim is assumed to be the result of the disturbed immunobiological processes, or perhaps to an interrupted association between the lymphocytic and macrophagic system. In the case of Lycurim-treated experimental dogs, the gradually increasing necrosis of leucocytes, which started in the early stage of the disease, may have been involved in se in the causation of increased phagocytosis in Kupffer cells, which finally led to an exhaustion of the macrophage system of the liver.

During the disease, replication of the virus, as well as signs of virus outflow from the cells was observed in a proportion of Kupffer cells. As a common result of immunosuppression and direct virus effect, the injury of endothelial cells in the sinusoids of the liver became more extensive with the progression of the disease, and there was an invasion of migrating red blood cells in the direction of the space of Disse. In the two Lycurim-treated animals the pathogenesis of haemorrhagic liver dystrophy was presumably stimulated, by a damage in the interferon-producing system, which allowed the pathogen to find free access to the hepatocytes across the sinusoidal walls. Therefore, in the last hours of the disease, an intensive virus replication occurred in hepatocytes. In dogs no. II and IV, virus replication in a great proportion of hepatocytes was favoured by the circumstance that with the destruction of the lymphoid tissue, even the specific humoral defence mechanism was suppressed. After all, by means of the artificial inhibition of cellular and humoral immune responses, we succeeded in producing acute viral hepatitis in two experimental dogs. The fact that dogs from the same litter and infected in identical way with equal doses of virus survived without any medical treatment supports the positive result of the experiment, and verifies the role of immunosuppression in the pathogenesis of the disease.

The pathogenesis of the reovirus-induced hepatitis of mice too, introduced by a damage of Kupffer cells (RUEBNER et al., 1964). Studies with mice treated with pathogenic virus and simultaneously with corticosteroids yielded results essentially similar to ours. In reovirus-infected mice treated with corticosteroids an increased mortality and an aggravation of hepatic changes were observed (STARR and POLLARD, 1958; MANSO et al., 1959; PIAZZA, 1965; HIRANO and RUEBNER, 1966).

It is worthy of note that in dogs with the fulminant form of VHD comes to an end within 1—3 days in general. In our experiments, the haemorrhagic liver dystrophy which resulted in the death of animals developed somewhat slower, *i.e.* within 5 days. This might perhaps be explained by the fact Lycurim affected primarily the humoral antibody-producing system, and the macro-

phagic system of the liver was injured only secondarily. It is worth mentioning, that in fulminant viral hepatitis the significant decrease in leucocyte count was considered by HORVÁTH et al. (1971) to be of diagnostic value clinically.

The fact, however, that in the experiment outlined in the foregoing, the individuals with active RHS system survived suggests that the adenovirus of VHD needs a helping factor for the lethal viral hepatitis to develop. Out of the factors that might come into consideration the role of immunosuppression is emphasized by these experiments. These findings might also explain why artificial infections result in frequent failure.

There are previous reports in the literature on lethal VHD after administration of various drugs (often anti-parasitic agents).

On the basis of these results it is assumable, that even in practical life, lethal viral hepatitis is not only the result of viral infection, but also the result of the impaired immune system of the infected animal. It is likely that known or unknown immunosuppressive effects of drugs are hidden in the background of deficient immunoreactivity.

Acknowledgement

We thanks the technicians, Mrs. PETHES and Mrs. TURCSÁNYI for their skillful assistance in the preparation of the light- and electron-microscopic slides and pictures.

Summary

Multiplication of the causative agent of viral hepatitis of dogs (VHD) was earlier shown to proceed mainly in the sinusoidal RHS cells of the liver, thus, it has been assumed that the seriousness of the Rubarth disease may been some relationship to the state of these cells.

In order to prove our hypothesis, 4 susceptible lettermate dogs at the age of four months were injected intramuscularly with comparable doses of the virus of VHD. Simultaneously with the infection, two of the dogs were administered a cytostatic drug (Lycurim) to damage the cellular immune system. Changes in the blood cell counts and those of liver in needle biopsy specimens were followed up. The counts of white blood cells (lymphocytes, in particular) markedly decreased, the Kupffer cells were damaged, and both of the dogs treated with the immunosuppressive drug died of acute VHD (acute liver dystrophy), whilst their two littermates survived.

The present study indicates that acute lethal VHD occurs primarily in the dogs with blocked liver cells of RHS.

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BIOTRANSFORMATION OF ZEARALENONE TO ZEARALENOL BY *CANDIDA TROPICALIS*

By

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Zearalenone [2,4-dihydroxy-6-(10-hydroxy-6-oxo-trans-1-undecenyl)-β-resorcylic acid-μ-lactone] is a well-characterized estrogenic metabolite of several species of *Fusarium*, most notably, several of the cultivars of *F. roseum* (sensu Snyder and Hansen).

This fungus metabolite is the cause of hyperestrogenism in swine and other reproductive disorders in swine, other livestock, and poultry (MIROCHA et al., 1971). With the exception of zearalenol, the naturally occurring metabolites of *Fusarium* which are closely related to zearalenone either have no estrogenic activity or have not been isolated in quantities great enough to test (PETERS, 1972; SHIPCHANDLER, 1975; HAGLER, et al., 1979).

Zearalenol [2,4-dihydroxy-6-/6,10-dihydroxy-trans-1-undecenyl/-β-resorcylic acid-μ-lactone], a close metabolic relative of zearalenone, was found in cultures of *F. roseum* 'Gibbosum' and 'Graminearum' by STIPANOVIC and SCHROEDER (1975). HAGLER et al. (1979) found 563 ppm of alpha-zearalenol and 8.000 ppm zearalenone in cultures of *F. roseum* 'Gibbosum', but concluded that beta-zearalenol was not present in the cultures. These authors showed that alpha-zearalenol was four times as active in the rat uterotropic bioassay as either zearalenone or beta-zearalenol. PETERS (1972) showed that alpha-zearalenol was a little more than three times as active as zearalenone and that beta-zearalenol was slightly less estrogenically active than zearalenone when fed to castrated female mice.

We wish to report the bioconversion of zearalenone to zearalenol at 32 °C and the conversion of ^{3}H-zearalanone [2,4-dihydroxy-6-(10-hydroxy-6-oxo-undecyl)-β-resorcylic acid-μ-lactone] to ^{3}H-zearalanol [2,4-dihydroxy-6-(6,10-dihydroxyundecyl)-β-resorcylic acid-μ-lactone] by *Candida tropicalis* at room temperature and to point out the possible contamination of feeds with zearalenol as well as the lack of methods of analysis for this compound in feeds. *Hansenula anomala* was able to metabolize zearalenone to zearalenol, though much less efficiently than *C. tropicalis*.

It was originally proposed that fermentation of zearalenone-contaminated maize or other grain with an organism such as *C. tropicalis* or *H. anomala* might detoxify the grain so that it could be used in animal feeding. Our data indicate that the zearalenone content is indeed reduced, but the zearalenone is replaced by a metabolite equally toxic.

Materials and methods

Stock cultures of *C. tropicalis* (NIH-K 10/1976) and *H. anomala* (NIH-K 9/1976) were maintained on Sabouraud agar slants under sterile mineral oil at 5 °C. Yeast was grown on 10 Sabouraud agar plate— for three to five days and then simply rubbed off the surface of the agar into 10 ml of sterile distilled water with a bent glass rod. The cells from the 10 plates were added aseptically to one litre of sterile broth and the flask shaken several times daily for a week. Viable counts were then obtained by dilution onto spread plates of Sabouraud agar. Culture broth was 2% peptone, 2% malt extract and 2% glucose per litre distilled water. The media were sterilized at 121 °C for 30 min before inoculation.

Zearalenone, 20 or 100 mg, in 20 ml of 96% ethanol was added to the 24-hr cultures of *C. tropicalis* or *H. anomala*. Incubation was aerobic at 32 °C and the flasks were shaken several times each day. Progress of transformation

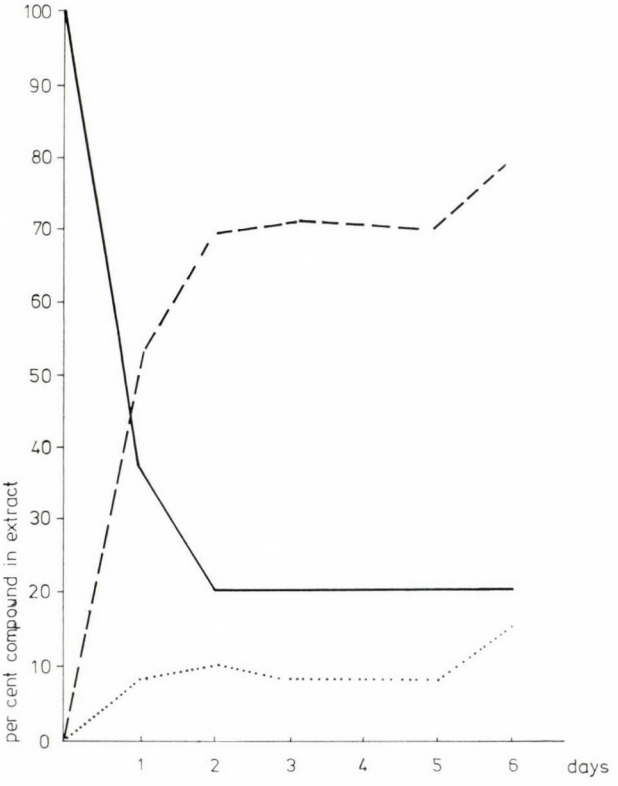

Fig. 1. Transformation of zearalenone to *alpha-* and *beta-*zearalenol by *Candida tropicalis*. Note that the predominant diastereomer is different from that produced by *Fusarium roseum* 'Gibbosum'. —— zearalenone; – – – *beta*-zearalenol; . . . . . *alpha*-zearalenol

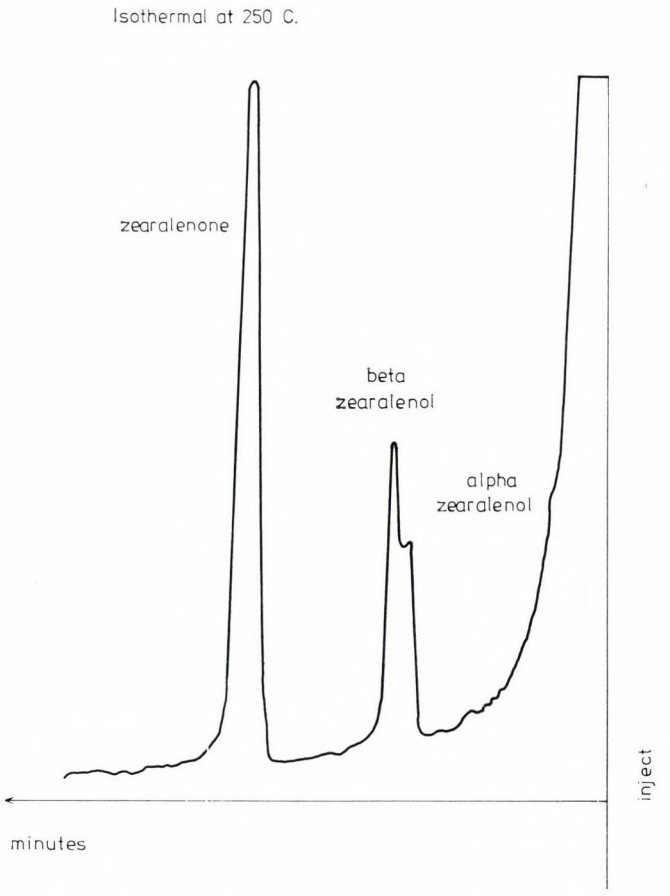

Fig. 2a. Gas-liquid chromatography of *alpha*, *beta*-zearalenol and zearalenone on 3% QF-1

of zearalenone to zearalenol was monitored by extracting 10-ml samples of culture broth with 10 ml of chloroform or extraction of 100 ml of culture broth, which had been centrifuged at 3000 rpm for 10 min with 100 ml ethyl acetate twice. Cultures were monitored generally every 24—48 hr. Chloroform extracts were discarded after thin-layer chromatography (TLC) while ethyl acetate extracts were evaporated and reserved for further analysis by TLC, gas-liquid chromatography (GLC), and high-performance liquid chromatography (HPLC). Quantification was by HPLC (Fig. 1).

Transformation was confirmed and monitored by TLC on 0.25 mm × × 20 cm × 20 cm silica gel 60 F-254 plates (E. Merck, Darmstadt, FRG developed in $CHCl_3$: CH_3OH (95 : 5, v/v). Developed plates were examined at 366 nm and 254 nm wave lengths and then sprayed according to the method of SARUDI (1974).

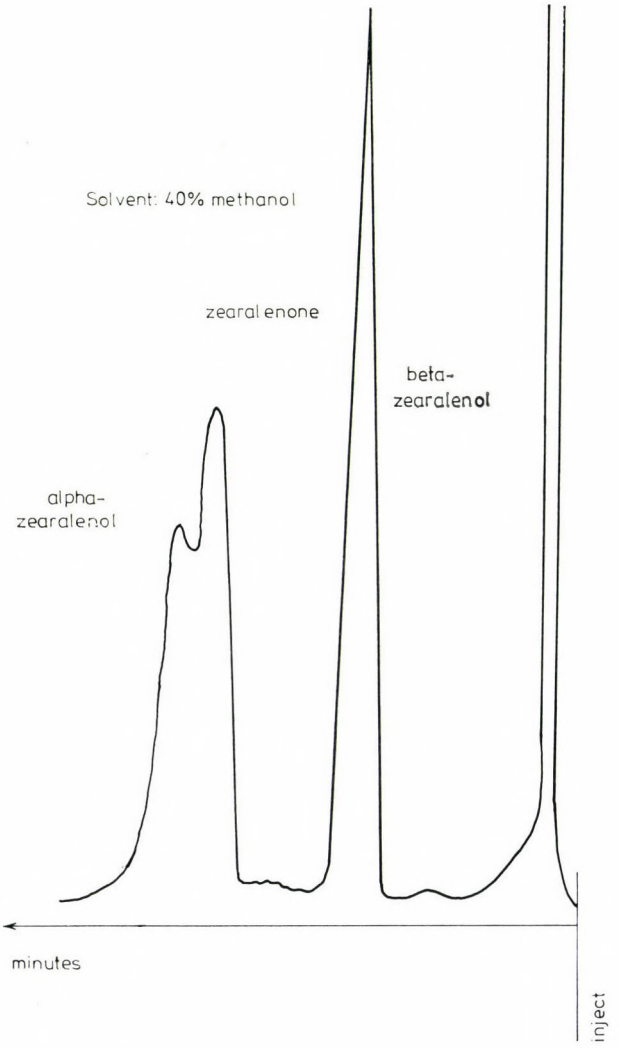

Fig. 2b. C_{18} HPLC of *alpha*, *beta*-zearalenol and zearalenone

A diastereomeric mixture of zearalenol prepared essentially by the method of SARUDI (1976) was used as a standard for GLC, TLC, and HPLC. The diastereomers of zearalenol were separated by GLC on 3% QF-1 and by HPLC on a reverse-phase column (Figs 2a and 2b).

Gas chromatograph was a Packard Model 7409 dual differential instrument fitted with flame ionization detectors (Packard Instrument Co., Downers, Grove, IL, USA). The liquid phase QF-1 (Supelco, Inc., Bellefonte, PA, USA) was coated onto 80—100 mesh silanized Gas-Chrom P (Carlo Erba, Italy) and packed into a 2-m long × 4 mm inside diameter glass column. Carrier gas was

argon at 60 ml/min and air and hydrogen were adjusted to 200 ml/min and 60 ml/min, respectively. Temperature was programmed from 150—250 °C at 6 or 8 °C/min or was isothermal at 250 °C. Injector and detector were operated at 250 °C. Extracts were silylated with a 5 : 1 mixture of BSA (bis-trimethyl-

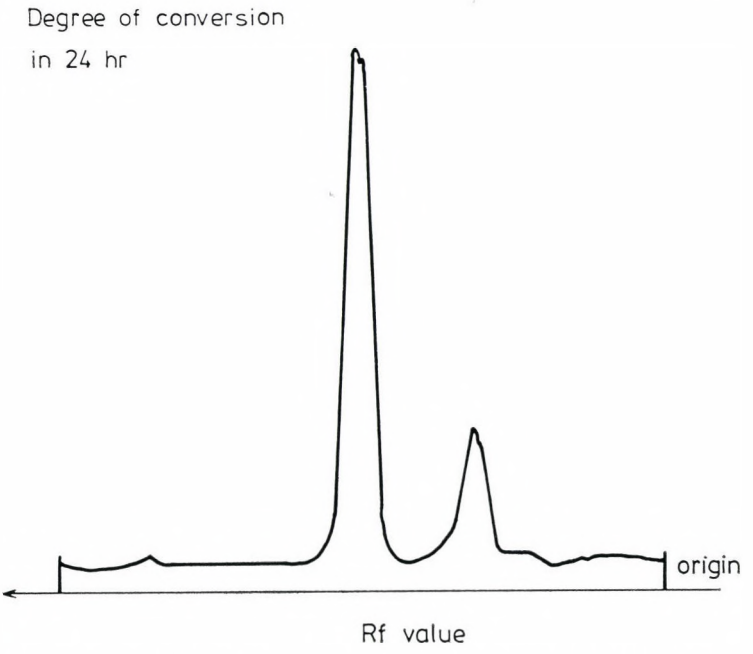

Fig. 3a. Distribution of radioactivity on chromatogram of extract from *Candida tropicalis* culture 24 h after adding ^{3}H-zearalanone. Experiments with ^{3}H-zearalanone were conducted at room temperature. Under these conditions conversion is slower

silylacetamide) and TMS (trimethylchlorosilane, Pierce Chemical Co., Rockford, IL, USA).

HPLC was accoplished with a Packard Model 8200 liquid chromatograph (Packard-Becker B. V., Delft, the Netherlands) equipped with an analytical C_{18} reverse phase column (Partisil PXS 10/25 ODS, Whatman Inc., Clifton, NJ, USA). Components were resolved with 40% methanol. Flow rate was 1.7—1.8 ml/min. Samples of 10 μl were injected.

Zearalenone was reduced to ^{3}H-zearalanone with pure $^{3}H_2$ over a palladium catalyst. ^{3}H-zearalanone was purified by TLC, and the R_f value in the solvent system above was the same as that of zearalenone. The specific activity of the zearalanone was 77 mCi/mg. Incubations and extractions were carried out as described above, but at room temperature. Distribution of radioactivity was determined by a Berthold TLC plate scanner (Berthold, Wildbad, FRG). (Figs 3a and 3b).

Mass spectra of the trimethylsilyl derivatives of components in culture extracts were obtained, using an LKB 9000 combination GC-MS (LKB-Producter AB, 25 Bromma, Sweden). Masses were calculated and printed by an on-line computer.

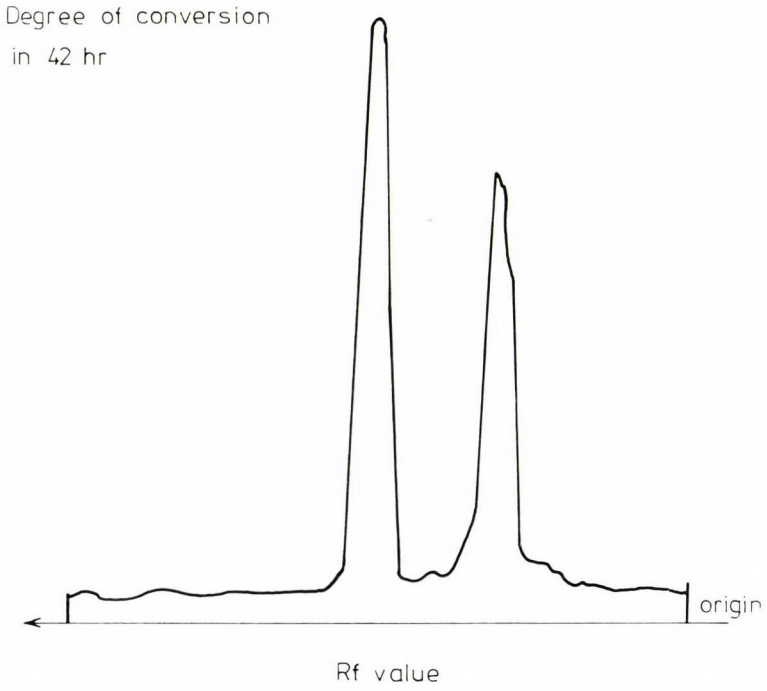

Fig. 3b. Distribution of radioactivity on chromatogram of extract from *Candida tropicalis* culture 42 h after ^{3}H-zearalenone had been added. Compare to conversion at 24 h

Results

Transformation of zearalenone to zearalenol by *C. tropicalis* was essentially complete by 48 hr with over 60% of the zearalenone converted by 24 hr. By the end of the experiment, 20% of the zearalenone had remained unchanged and 80% had been converted to zearalenol. Zearalenol was present in the extracts as about 89% beta-zearalenol and 11% alpha-zearalenol. Little, if any, conversion occurred after 48 hr, even though incubation was continued for 6 days after addition of the zearalenone. These results are depicted in Fig. 1. Analyses of ethyl acetate extracts of sterile broth amended with zearalenone by TLC and HPLC revealed no change in the zearalenone over the duration of the experiment (not shown). Analyses of extracts of sterile unamended broth and of the broth culture of *C. tropicalis* with no zearalenone added, in the same manner over the length of the experiment as the treated cultures, revealed no components in either case which interfered with analysis by TLC or HPLC.

R_f values in the solvent system used were 0.58 and 0.35 for zearalenone and zearalenol, respectively. Separation of zearalenone and alpha- and beta-zearalenol by GLC and HPLC is shown in Fig. 2. HPLC analyses were occasionally checked by GLC. Both GLC and HPLC revealed that the major components in all ethyl acetate extracts of zearalenone-amended cultures or broth were zearalenone and/or zearalenol. GLC and HPLC under these conditions afforded the resolution necessary to unambiguosusly identify not only the diastereomers of zearalenol, but also the proportions of each produced by *C. tropicalis* from zearalenone. Quantification, therefore, was possible directly from the ethyl acetate culture extracts. Peak areas were calculated by multiplying the height of the peak by the width at one-half the peak height.

The dilution series revealed that on the order of 10^7 *C. tropicalis* cells per ml were present in the treated and control cultures.

No contamination of cultures with bacteria or fungi was noted in plates streaked for isolation of contaminating organisms.

H. anomala exibited an identical pattern of transformation of zearalenone to zearalenol, though it was much less efficient than *C. tropicalis* in this conversion (unpublished data).

The analyses of extracts from experiments in which tritiated zearalenone, that is radioactive zearalanone was added to *C. tropicalis* cultures by scanning the radioactivity on a thin layer chromatogram revealed only one new peak of activity (Figs 3a and 3b). We concluded that the transformation product was zearalanol.

Gas-liquid chromatography—mass spectrometry (GLC-MS) of the conversion product of zearalenone was zearalenol, since on comparison of mass spectra the component in the extract had a spectrum identical to that of standard TMS-zearalenol (unpublished data). GLC-MS was not performed on the radioactive extracts.

Conclusions

The *C. tropicalis* isolate used in these biotransformation experiments was from the Central Food Industrial Research Institute, Budapest (PALYUSIK, 1973) and the *H. anomala* strain was isolated from high-moisture corn which had been stored under plastic in a high-carbon-dioxide atmosphere according to the proposed storage method of the University of Keszthely, Hungary. We suggest the potential for conversion of zearalenone to zearalenol in zearalenone-contaminated grain in a storage situation such as this.

Probably every analyst for mycotoxins has analysed samples of feeds associated with definite clinical cases of hyperestrogenism and has obtained results which were negative for zearalenone and diethylstilbestrol. Some, at least, of the cases could perhaps be diagnosed if an analysis for zearalenol were included.

It is very striking that beta-zearalenol, the primary metabolite of zearalenone from *C. tropicalis* and swine (PATHRE et al., 1978) is a diastereomer different from that produced by *F. roseum* 'Gibbosum' (HAGLER et al., 1979). Since the diastereomers differ with origin, it might be possible to determine the origin of the zearalenol if methods suitable for resolving the diastereomers are used.

Summary

In aerobic liquid culture at 32 °C, *C. tropicalis* reduced 80% of the added zearalenone to zearalenol; with beta-zearalenol, the major product (89%) and alpha-zearalenol (the diastereomer found in cultures of zearalenone-producing strains of *F. roseum* 'Gibbosum' (the minor product) 11% of the total zearalenol). Only 20% of zearalenone added remained unchanged in the culture and no other metabolites were found. Conversion was complete in 48 hr and the greatest percentage of conversion took place in the first 24 hr.

The reported estrogenicity of beta-zearalenol is about equal to that of zearalenone while that of alpha-zearalenol is about four times that of zearalenone. Analyses were performed with GLC on 3% QF-1 and HPLC on a C_{18} reverse-phase column and confirmed by mass spectrometry. The results suggest that fermentation of zearalenone-contaminated feedstuff would not be a useful means of detoxification. Further, the possibility of conversion of zearalenone to zearalenol on a fairly large scale in feedstuffs by the natural microflora or microfauna emphasize the need for development of methods of analysis for this metabolite. Metabolism of ^{3}H-zearalanone to ^{3}H-zearalanol by *C. tropicalis* at room temperature with no other radioactive metabolites lends strength to the conclusion that no other metabolites are formed from zearalenone by *C. tropicalis*.

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EXPERIMENTAL STUDIES ON EARLY WEANED LAMBS

III. HAEMATOLOGICAL APPRAISAL OF PROTEIN AND AMINO-ACID NUTRITION

By

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Assessment of the amino acid needs of lambs reared on milk replacer has been attempted by analysis of either nitrogen metabolism or blood parameters. Multiple evidence has been presented of the impact of dietary proteins on the plasma amino-acid level. NIMRICK et al. (1970) established that the essential amino-acid need of lambs follows the sequence methionine, lysine and threonine, and that the plasma amino-acid level of the growing lamb shows a direct correlation with the plane of amino-acid nutrition. BERGEN and POTTER (1975) investigated the effect of dietary protein content on the plasma and tissue levels of amino acid in the sucking lamb. Elevation of the milk substitute's protein and amino-acid content was followed by a distinct rise of essential amino acid level in the blood plasma, while reduction of the dietary level of both nutrients by a distinct drop thereof. WAKELING et al. (1970) assessed the protein requirement of sheep by following up the fluctuation of free amino-acid level in the blood plasma after duodenal infusion of different quantities of methionine or lysine, and concluded that both represented the limiting amino acids. KOSAROV et al. (1975) established that lysine is of primary importance in ovine feeds. They proposed to adjust the lysing content of milk substitutes employed for artificial lamb rearing to a level corresponding to that in ewe's milk. KORSHUNOV et al. (1974) regarded both methionine and lysine as essential amino acids for sheep, on the basis of a direct correlation demonstrated between intake and plasma levels of these amino acids.

JUHÁSZ and KIRÁLY (1961) studied the development ruminant digestion, first in calves. On subsequent experiments of lambs, those concerned with development of the digestive function and related metabolic alterations (SZEGEDI et al., 1980) as well as with nitrogen and amino-acid retention under artificial rearing conditions (SZELÉNYI-GALÁNTAI et al., 1980) were reported.

Investigations into the amino-acid metabolism of early-weaned lambs artificially reared on milk replacer are described in this paper. The main aspects of this study were the influence of methionine and lysine supplementation on growth, the correlation of dietary and blood amino-acid levels, and the impact of the plane of protein and amino-acid nutrition on certain blood parameters.

Materials and methods

Three groups of Merino lambs were used for the experimental study of protein and amino-acid nutrition. The young animals had been maintained on the ewe for two days to take up the colostrum, and were subsequently weaned and put on milk replacer (PHYLAXIA, Budapest) supplied by pail-feeding

on five occasions daily (at 7 a.m., 10 a.m. and 1, 4 and 7 p.m.). In addition to the milk substitute ration, drinking water, and a pelleted lamb prestarter formulated by us were made available *ad libitum*.

In the different groups, the milk replacer was withdrawn on different schedules, and was fed either with or without amino-acid supplement.

In group I, the plain milk replacer ration was reduced gradually, while the prestarter was made available *ad libitum*.

In groups II and III, the milk substitute was equally withdrawn abruptly, but while group-II lambs had received the plain substitute, in group III it had been supplemented with synthetic L-lysine and DL-methionine, adjusted to the level usual in ewe's milk. The prestarter fodder mixture was made available *ad libitum* in these groups too.

Feed composition, constituents and consumption, as well as the diet regimes employed in the three groups were described in detail in the second part of this publication series (SZELÉNYI-GALÁNTAI et al., 1980).

The intake and elimination of protein, nitrogen, lysine and methionine were determined daily over five test periods, each five days long, until 40—42 days of age. During the period of study, the lambs were kept singly in metabolic cages to make possible the individual determination of feed consumption, and of urine and faeces discharge.

The haematological appraisal of protein and amino-acid nutrition was based on three blood samplings from three animals per group in each test period, *i.e.* on a total of 45 samples. Blood was always withdrawn from the jugular vein, 3 hrs after feeding.

The blood samples, heparinized at withdrawal, were immediately centrifuged. The plasma was analysed for free lysine and methionine, total amino acid-nitrogen, total protein and non-protein nitrogen (NPN). Plasma lysine and methionine were isolated as proposed by STEIN and MOORE (1954). The amino-acid components of deproteinized plasma were determined with a BioCal 200 typ. amino-acid analyser, as described MOORE et al. (1958) and DÉVÉNYI (1969).

Plasma total protein was determined according to PHILLIPS et al.; NPN by the titrimetric procedure of RAPPAPORT and EICHORN; total amino acid-N by FOLIN and DANIELSON's method. All described in a manual edited by BÁLINT (1962).

Results

Group I

The average daily intake of lysine was 7.1—3.6 g from the first to the fifth test period. This figure includes the lysine derived from both milk replacer and prestarter. The plasma lysine level changed from the initial 19.9 ± 6.93 to 13.6 ± 1.35 μmol/100 ml during the third test period. The average daily

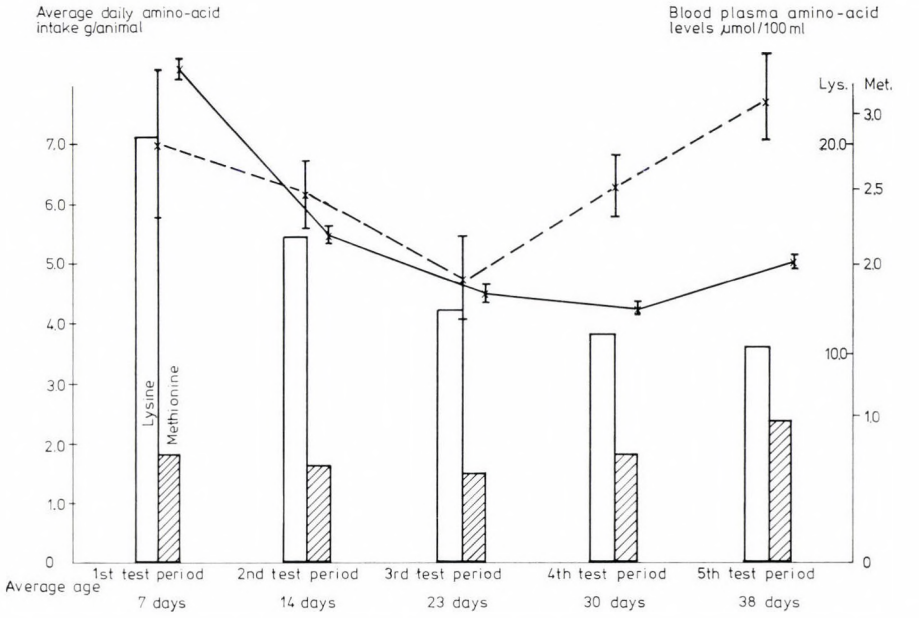

Fig. 1. Dietary intake and plasma levels of lysine and methionine in group I. ×— —× lysine; ×——× methionine

intake of methionine fell from the 1.8 g measured during the first test period to 1.5 g measured during the third one; intakes in the fourth and fifth period were 1.8 and 2.0 g, respectively. The initial plasma methionine level of 3.2 ± ± 0.28 decreased to 1.8 ± 0.77 μmol/100 ml during the third test period, then rose again to 2.0 ± 0.42 over the fourth and fifth periods (Fig. 1).

The other tested blood parameters showed the following changes:

Plasma total protein fluctuated between 4.8 ± 0.70 and 5.8 ± 0.57% w/v between 2 and 30 days of age, and subsequently rose to 7.1 ± 0.24% w/v by the end of the experiment.

The total amino-acid nitrogen content of the plasma decreased from the initial 9.8 ± 1.28 to 6.0 ± 0.57 mg/100 ml until the fourth test period, then rose slightly to 6.6 ± 0.97 mg%. The plasma NPN level fell gradually from 17.5 ± 2.04 to 9.1 ± 1.67 mg/100 ml by the fourth test period, followed by a slow rise to 10.8 ± 0.80 mg/100 ml during the fifth period.

Group II

Average daily lysine intake from milk replacer and solid feed was 5.7, 6.7, 7.4, 3.4 and 0.9 g, respectively, in the five test periods. The respective average daily methionine intakes were 1.5, 1.8, 2.0, 1.0 and 0.7 g. The plasma

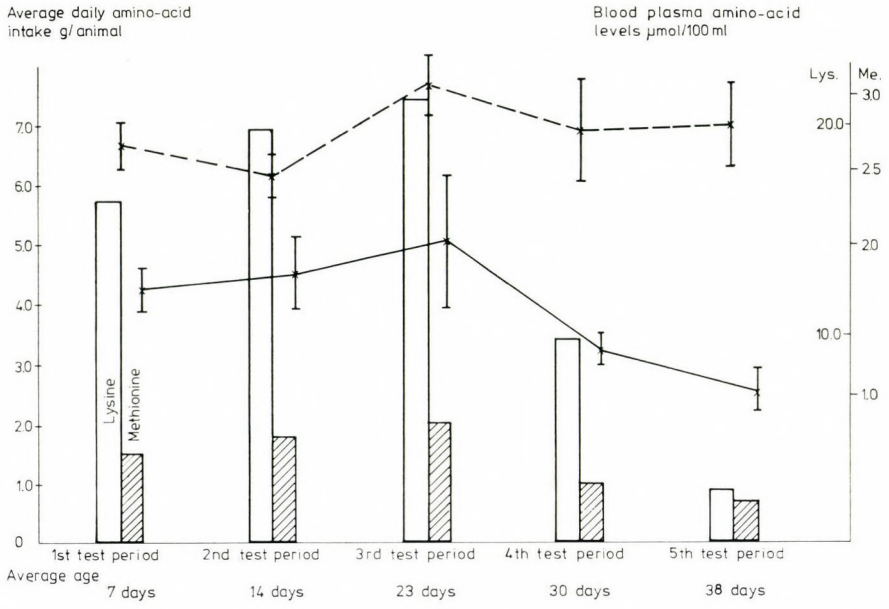

Fig. 2. Dietary intake and plasma levels of lysine and methionine in group II. ×— —× lysine; ×——× methionine

lysine level reached its maximum, 21.9 ± 1.65 μmol/100 ml, in the third test period, then fell to 19.8 ± 0.22 and 19.6 ± 1.80 μmol/100 ml, respectively, in the fourth and fifth period. The plasma methionine level was 1.7 ± 0.36, 1.8 ± 0.54 and 2.1 ± 0.45 μmol/100 ml in the first three test periods, then tended to decrease to 1.3 ± 0.1 and 1.1 ± 0.15 μmol/100 ml in the fourth and fifth ones (Fig. 2).

Plasma total protein content was lowest, 6.9 ± 0.87 mg%, in the third test period, but it did not change significantly throughout the experiment.

Plasma total amino-acid nitrogen fell to 6.2 ± 0.12 mg% in the third period, followed by a drop of plasma NPN to 18.4 ± 2.77 mg/100 ml.

Group III

The lambs of this group received lysine and methionine supplemented milk replacer from the second test period (7 days of age) on. This was followed by a temporary increase in the amino acid intake which, however, began to decrease steadily owing to reduced feed consumption after withdrawal of the milk substitute from the third test period on. Average daily lysine intake was 6.8, 9.3, 7.3, 2.5 and 1.0 g, respectively, in the five test periods. The respective average daily methionine intake was 1.8, 2.8, 2.2, 0.9 and 0.8 g (day)animal. The plasma lysine and methionine levels tended to

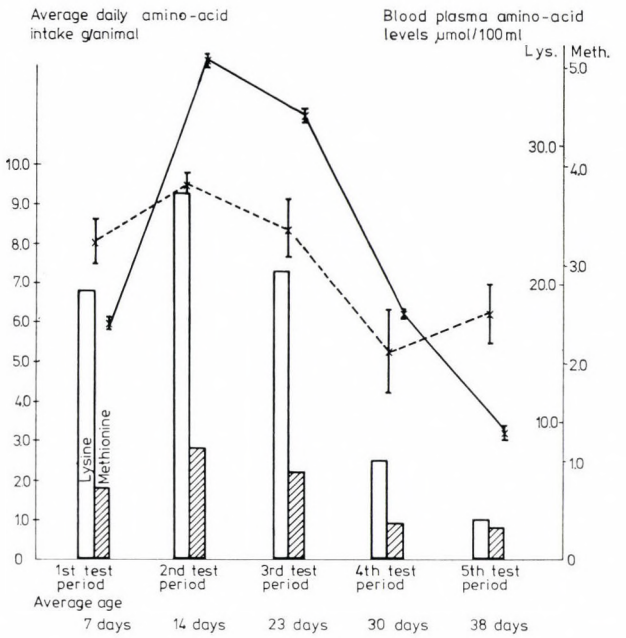

Fig. 3. Dietary intake and plasma levels of lysine and methionine in group III. ×— —× lysine; ×——× methionine

rise, especially during the second test period, in which lysine reached a maximum of 27.4±0.40 μmol/100 ml, but then it fell to a minimum of 15.4±1.38 μmol/100 ml in the fourth period. The methionine level reached its peak, 5.6±0.14 μmol/100 ml, in the second test period, and fell to a minimum of 1.3±0.11 μmol/100 ml in the fifth one (Fig. 3).

The other blood parameters, total serum protein, total amino-acid nitrogen and NPN, showed minima of 6.8±0.32% w/v, 6.7±0.50 mg/100 ml and 13.7±1.48 mg/100 ml respectively, in the third test period.

Discussion

Parallel with the reduction of the milk replacer ration, the group I lambs ingested a sufficient quantity of solid prestarter to ensure undisturbed growth. The plasma amino acid level followed the quantitative relations of dietary amino-acid supply until the third test period; after day 23 the plasma lysine level strikingly deviates from the parallel running.

In group II, abrupt withdrawal of the milk substitute, and inadequate intake of solid prestarter accounted for a drop of the plasma lysine and me-

thionine levels after day 23. Decrease of the amino-acid intake was more pronounced with methionine than with lysine.

In consequence of lysine and methionine supplementation lambs of group III showed an increase of the plasma lysine and methionine levels over the group I and group II lambs between days 14 and 23, after which the abrupt withdrawal of the supplemented milk substitute accounted for a considerable concentration decrease of both amino acids in the plasma. As the lambs deprived of the milk substitute failed to take in a sufficient quantity of prestarter, their protein and amino-acid needs were temporarily not satisfied. After 30 days of age, when the intake of the prestarter began to normalize, the plasma lysine level tended to rise again.

It follows from the experimental observations that fluctuation of the blood amino-acid level of sucking lambs show a parallelism with dietary amino acid intake until 23 days of age, but deviates from the latter thereafter. This accords well with our finding (SZEGEDI et al., 1980) that the development of ruminal digestive function becomes completed by 21—24 days of age. After this period notable alterations of protein and amino-acid metabolism did occur in all three experimental groups.

Our experimental observations indicate that gradual withdrawal of the milk replacer and simultaneous increasing intake of the solid feed ration do not interfere with the enhanced metabolic rates of protein and amino acid in artificial lamb rearing, for under this schedule the amino-acid intake is able to furnish the required amounts during the period of charying diet. Since with a not fully developed ruminal digestive mechanism, solid feed intake cannot compensate for the amino-acid supply lost through the withdrawal of milk substitute, an undisturbed growth cannot be expected. As the mechanical and chemical stimuli associated with solid food intake stimulate the functional development of the rumen, lambs should be offered gradually increasing supplies of fodder prestarter.

The correlation between the dietary amino-acid intake and plasma amino-acid levels of sucking lambs was pursued by mathematical-statistical analysis. Equations of regression were calculated and plotted out graphically in Fig. 4.

The regression equation for lysine:

$$y = 0.408x + 3.09 \quad n = 15 \quad r = 0.5717$$

was significant at $P < 0.001$.

The equation for methionine:

$$y = 0.364x + 0.83 \quad n = 15 \quad r = 0.6583$$

was significant $P < 0.01$.

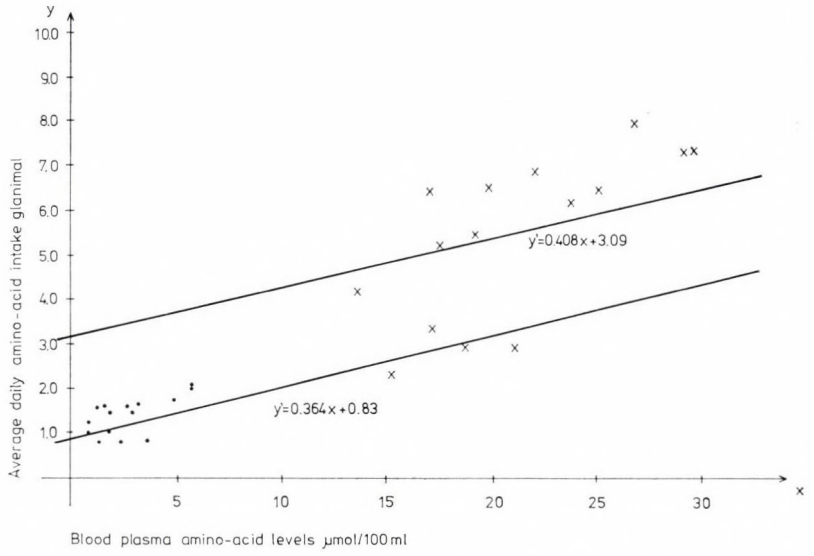

Fig. 4. Correlation between intake and plasma levels of lysine and methionine. xxx, r = 0.5717; y' =0.408x + 3.09; . . . r = 0.6583; y' = 0.364x + 0.83

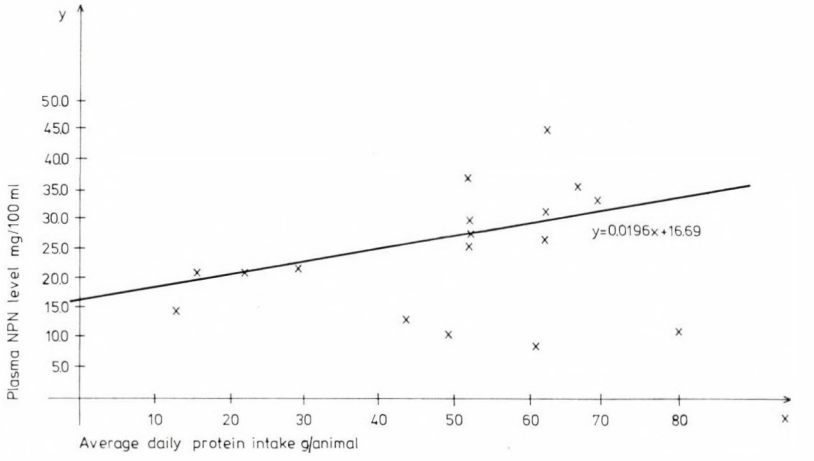

Fig. 5. Correlation between protein intake and plasma non-protein nitrogen level. r = 0.6118; y = 0.0196x + 16.69

The correlation coefficients and the regression equations uniequivocally indicate a close correlation between the dietary and plasma levels of lysine and methionine.

Correlation was also pursued between the dietary protein supply and plasma NPN level. Earlier experiments on sheep (LEWIS, 1957) and fattening bullocks (JUHÁSZ and KIRÁLY, 1961) have indicated a parallelism of plasma

NPN with protein nutrition. The correlation demonstrated under the given conditions of experiment can be characterized by the regression equation

$$y = 0.0196x + 16.9; \quad n = 15; \quad r = 6118$$

which is plotted out graphically in Fig. 5.

The correlation coefficient and the equation showed significant correlation between the two parameters ($P < 0.01$).

The interrelationships emerging from biometric evaluation indicated that blood analysis is in fact cunclusive of protein and amino-acid nutrition im lambs. Rapid chemical and biochemical tests proved to be suitable for the analytical approach. ARMSTRONG and ANNISON (1973) have arrived at a similar conclusion. Accordingly the plasma amino-acid level was conclusive of the amino-acid nutrition of lambs under well-controlled conditions of experiment.

Summary

Protein and amino-acid nutrition were studied in three groups of early-weaned, artificially reared lambs under different schedules of milk replacer supply and weaning time.

The level of amino-acid nutrition was assessed from free lysine and methionine, as well as total amino-acid nitrogen contents of blood samples taken 3 hr after feeding.

In group I, gradual withdrawal of the milk replacer was successfully coordinated with the rising intake of a solid prestarter diet as protein and amino-acid source, resulting in undisturbed absorption and incorporation of these essential nutrients during the period of diet change. Fluctuations of the blood parameters followed the quantitative alterations of dietary protein and amino-acid supplies until 23 days of age. A similar tendency was recognized in group II, but at different quantitative relations owing to the dissimilar feeding schedule. In group III amino-acid supplementation of the milk replacer from 7 days of age accounted initially for a considerable rise of the plasma lysine and methionine levels which, however, fell markedly upon abrupt withdrawal of the milk replacer, and did not normalize until the onset of the ruminal digestive function and gradual increase of solid food intake at a later time.

The correlation between the quantities of ingested lysine and methionine and their plasma concentrations was significant.

The plane of protein nutrition was assessed from the fluctuations of plasma total protein and non-protein nitrogen (NPN) contents, on the grounds of significant correlation between intake and plasma level.

The experimental observations have shown that the uncomplicated early weaning of lambs presupposes an undisturbed protein and amino-acid nutrition. Under the given conditions of experiment gradual withdrawal of the milk replacer from 12 to 32 days of age made possible the intake of the required protein and amino acid resources by incrasing consumption of solid feed during the critical period.

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EXPERIMENTAL STUDIES ON EARLY WEANED LAMBS

IV. ENERGY AND GAS METABOLISM

By

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Problems in early weaning and artificial rearing of lambs propted us to study gastric digestion and internal metabolism (SZEGEDI et al., 1979), nitrogen and amino-acid metabolism (SZELÉNYI-GALÁNTAI et al., 1980) and protein and amino-acid nutrition (JÉCSAI et al., 1980) in lamb reared on milk replacer diets. Studies on energy and gas metabolism are reported in this paper.

A milk replacer, formulated as proposed in the literature, was examined for value as energy resource, with special regard to its effect on weight gain. Further, changes in gas metabolism were followed up under experimental conditions.

The composition of ewe's milk and the related literary data have unequivocally shown that the growing lamb requires high dietary supplies of protein and energy. Authors generally agree that at an average protein to energy ratio, the lipid contant of the milk replacer should be preferably kept at about 20%. Several authors (MOLENAT and THERIEZ, 1971; OWEN and DAVIS, 1970; CHIOV and JORDAN, 1973) have proposed that a replacer containing 24—26% protein and about 20—25% fat relative to dry matter should be supplied to newborn lambs in a daily ration of 250—300 g to ensure a daily weight gain of 0.2—0.3 kg.

It follows from the foregoing that the nutritional aspects of early lamb weaning have been fairly well established. Little is known, however, on the physiological aspects of the problem.

Methods

The conditions and scheme of the experiments were the same as described in part II (SZELÉNYI-GALÁNTAI et al., 1980) of this publication series.

The quantitative relations of dietary energy intake and excretion of energy sources with the urine and faeces were determined daily. Urine samples were acidified with H_2SO_4 before evaporation, to prevent loss of N. It is known that the energy and N contents of the urine are closely interrelated (Es et al., 1971). The dietary, faecal and urinary energy levels were determined by calorimetry, in a Berthelot-Mahler apparatus, according to the standard prescriptions valid in Hungary (MNOSZ 700—55).

The utilizable energy was calculated from faecal and urinary excretion only, for the digestion of the young lamb functions, as in a monogastric animal, without notable gas formation.

The maintenance energy requirement was assessed from alterations of gas metabolism. The measurements were performed twice daily, in the interim between feedings, possibly in the same hour, in standing animal.

A type MG—4 Kipp-zonen diapherometer was used. The lambs were transferred to heat-insulated respiration boxes. The ambient temperature was maintained about 20°C during the period of measurement, and the maintenance energy requirement was calculated from records of O_2 consumption, as proposed at the 2nd Symposium on Energy Metabolism in Wageningen. We used THORBEK's formula (1975) with the modification that we relied on our own measurements instead of N values for assessment of the urine's energy content. The quantity of energy represented by the protein component of weight gain was calculated on the basis of the caloric energy equivalent of protein (1 g = 5.32 kcal).

The amount of energy required for the synthesis of 1 g protein was assessed from the percentual participation of body protein in the energy retention

Results

The lambs in group I received 1459 and 4147 Kcal energy in each litre of milk replacer and each kg of solid feed, respectively. Lambs of groups II and III, kept on an identical diet regime, received 1414 and 3870 Kcal energy in each litre and kg of replacer and solid, food, respectively.

In group I, the milk replacer was withdrawn gradually on a continuous schedule until the end of the third experimental period; by that time the animals had taken up a considerable amount of solid food in their daily ration. This accounted for a steady increase in energy intake, which consequently surpassed the initial value during the third stage of the experiment.

The milk-replacer ration of the group II and group III lambs was reduced abruptly, from the third and second stage of experiment, respectively. The animals failed to increase their solid food intake to a compensatory degree; thus an abrupt drop in energy consumption followed (Table I), accounting for a corresponding reduction of digestible energy supply (Table II).

In those experimental stages in which the entire ration, or the bulk of it, consisted of milk replacer, about 95% of the total energy intake was utilized as digestible energy (Table III).

The value of a diet depends on its digestible (utilizable) energy content. The utilizable energy resources available to the lambs for maintenance and production (weight gain in this instance) during the different experimental

Table I

Average daily energy consumption (Kcal) during the subsequent stages of the experiment

Experimental groups	I	II	III
Stage 1	1523 ± 152.8	1183 ± 389.4	1435 ± 100.8
Stage 2	1209 ± 152.2	1488 ± 227.2	1337 ± 197.0
Stage 3	1150 ± 243.9	1569 ± 189.2	1079 ± 148.0
Stage 4	1379 ± 396.2	654 ± 100.2	453 ± 230.0
Stage 5	1785 ± 329.5	442 ± 281.0	467 ± 368.2

Table II

Average daily intake of digestible energy (Kcal)

Experimental groups	I	II	III
Stage 1	1440 ± 146.7	1128 ± 382.5	1364 ± 75.6
Stage 2	1204 ± 91.9	1414 ± 219.3	1277 ± 72.5
Stage 3	1026 ± 245.9	1496 ± 188.1	1032 ± 56.3
Stage 4	1198 ± 326.9	607 ± 96.8	425 ± 179.2
Stage 5	1450 ± 269.9	386 ± 250.3	385 ± 282.2

Table III

Digestible energy consumption in per cent of the total energy supply

Experimenal groups	I	II	III
Stage 1	94.5	94.2	95.2
Stage 2	96.5	95.0	95.5
Stage 3	93.2	95.3	95.6
Stage 4	86.9	92.8	93.8
Stage 5	81.0	87.3	82.5

Table IV

Average daily intake of utilizable energy (Kcal)

Experimental groups	I	II	III
Stage 1	1390 ± 141.8	1100 ± 374.7	1333 ± 75.6
Stage 2	1176 ± 100.6	1371 ± 207.5	1234 ± 79.6
Stage 3	1026 ± 245.9	1430 ± 177.7	999 ± 56.5
Stage 4	1157 ± 317.9	556 ± 99.9	395 ± 106.3
Stage 5	1399 ± 265.8	358 ± 257.8	360 ± 286.4

periods are shown in Table IV, and the digestible energy in percentage of the total energy supply in Table V.

The absolute quantity of the energy passed with the urine did not change during the period of observation (Table VI), but the relative excretion of utilizable energy increased about twofold during the periods of inadequate feed intake (Table VII, Fig.1).

The maintenance energy requirement for each experimental group is shown in Table VIII. (The great individual variation found was due to differences in body weight.) The maintenance energy requirements calculated in per cent of the utilizable energy are shown in Table IX.

Table V

Utilizable energy consumption in per cent of the total energy supply

Eperimental groups	I	II	III
Stage 1	91.2	92.9	92.9
Stage 2	92.3	91.1	92.3
Stage 3	89.2	91.1	92.6
Stage 4	83.9	85.0	87.2
Stage 5	78.4	81.0	77.0

Table VI

Average daily excretion of energy with the urine (Kcal)

Experimental groups	I	II	III
Stage 1	50	28	31
Stage 2	28	43	43
Stage 3	46	66	33
Stage 4	41	51	30
Stage 5	51	28	25

Table VII

Average urinary excretion of energy in per cent of the total energy supply

Experimental groups	I	II	III
Stage 1	3.5	2.5	2.3
Stage 2	2.4	3.1	3.4
Stage 3	4.4	4.4	3.2
Stage 4	3.4	8.4	7.2
Stage 5	3.5	7.3	6.5

Table VIII

Average maintenance energy requirement per day (Kcal)

Experimental groups	I	II	III
Stage 1	687 ± 46.4	548 ± 65.1	723 ± 105.0
Stage 2	786 ± 70.1	748 ± 357.2	752 ± 98.0
Stage 3	791 ± 115.3	804 ± 182.6	810 ± 57.6
Stage 4	829 ± 76.4	762 ± 161.9	700 ± 79.2
Stage 5	896 ± 104.2	723 ± 105.0	883 ± 142.4

Table IX

Maintenance energy requirement in per cent of the total utilizable energy

Experimental groups	I	II	III
Stage 1	48.7	49.8	44.7
Stage 2	66.8	54.5	60.9
Stage 3	77.0	56.2	81.0
Stage 4	71.6	—	—
Stage 5	64.0	—	—

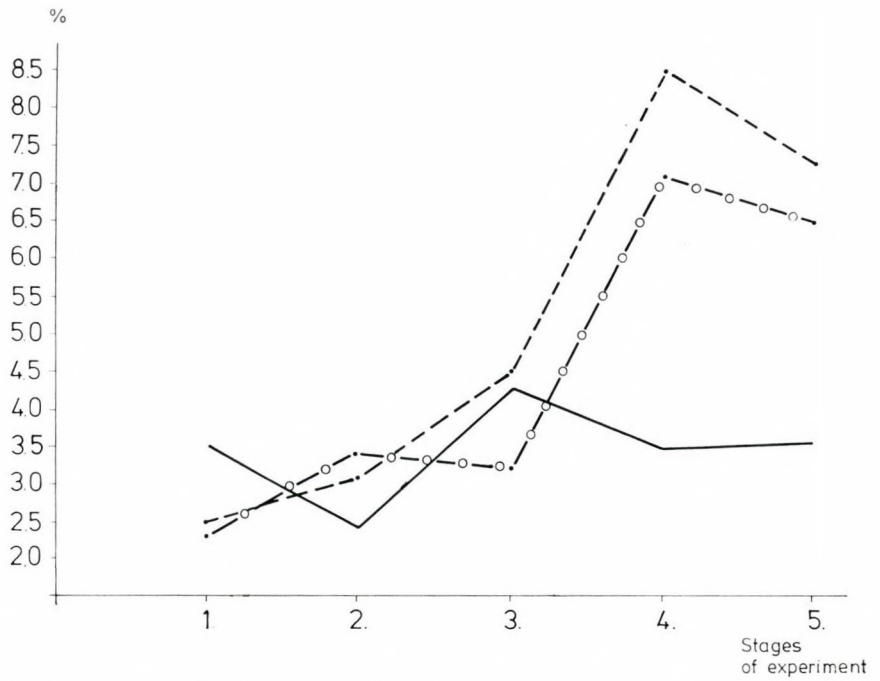

Fig. 1. Energy excreted with the urine in per cent of utilizable energy. —— Group I; – – – Group II; — ○ — Group III

Obviously, the absolute maintenance energy requirement of an animal increases parallel with the latter's weight gain, while at the same time the relative requirement calculated for 1 kg live weight tends to decrease along with the hourly heat loss. (Tables X and XI).

The maintenance energy requirement always represents a substantial part of the energy consumption, being the greater the lesser the energy intake. It follows that the energy reserves also become diminshed, as shown in Table XIII.

Table X

Maintenance energy requirement relative to 1 kg live weight (Kcal/day)

Experimental groups	I	II	III
Stage 1	107 ± 10.3	108 ± 6.9	109 ± 2.4
Stage 2	96 ± 11.2	102 ± 3.6	101 ± 4.6
Stage 3	85 ± 15.1	89 ± 7.7	100 ± 5.1
Stage 4	80 ± 7.1	80 ± 6.2	80 ± 6.2
Stage 5	76 ± 8.5	78 ± 3.6	77 ± 6.1

Table XI

Average heat loss per hour (Kcal/kg bwt)

Experimental groups	I	II	III
Stage 1	4.46 ± 0.80	4.51 ± 0.09	4.55 ± 0.08
Stage 2	4.03 ± 0.68	4.27 ± 0.15	4.19 ± 0.17
Stage 3	3.58 ± 0.90	3.72 ± 0.31	3.66 ± 0.40
Stage 4	3.36 ± 0.38	3.30 ± 0.26	3.35 ± 0.25
Stage 5	3.23 ± 0.40	3.55 ± 0.08	3.23 ± 0.14

Table XII

Average stored energy (Kcal/day/group)

Experimental groups	I	II	III
Stage 1	722 ± 154.6	551 ± 299.9	728 ± 84.0
Stage 2	385 ± 146.5	626 ± 59.9	482 ± 72.4
Stage 3	264 ± 225.0	622 ± 184.9	188 ± 75.4
Stage 4	349 ± 300.6	—205 ± 137.2	—288 ± 156.0
Stage 5	507 ± 253.0	—365 ± 202.7	—299 ± 164.7

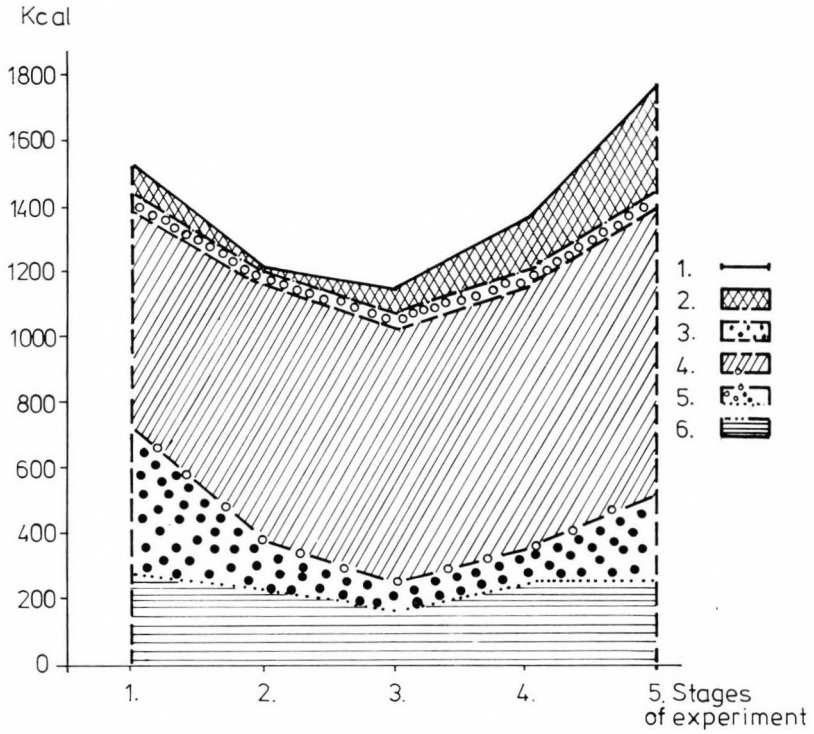

Fig. 2. Fluctuation of the energy balance in group I. 1. Total available energy; 2. Energy excreted with the faeces; 3. Energy excreted with the urine; 4. Maintenance energy requirement; 5. Lipid-associated energy reserve; 6. Protein- associated energy reserve

During the first stage of experiment 45—50% of the utilizable energy was used up for maintenance in each experimental group, while the rest was reserved in the form of weight gain. In the later stages of experiment during which the energy intake was decreasing, while the maintenance requirement was increasing owing to weight gain, the energy reserves tended to diminish.

During the fourth and fifth experimental periods the lambs of groups II and III already showed a negative energy balance. The values of energy retained in association with body protein are shown in Table XIII, and in percentage of total energy reserve in Table XIV.

The absolute quantity of protein-associated energy reserves tended to decrease as the experiment was progressing, while relative to the total reserve is showed an increasing tendency to the total reserve is showed an increasing tendency (Table XIV, Fig. 2).

Protein synthesis and a corresponding reservation of energy also took place in face of a negative energy balance (Table XV, Figs 3 and 4).

The values of utilizable energy used up for the synthesis of 1 g protein are shown in Table XVI.

Table XIII

Average energy reserves associated with body proteins (Kcal/day/animal)

Experimental groups	I	II	III
Stage 1	275 ± 49.6	212 ± 76.9	266 ± 28.8
Stage 2	221 ± 154.9	247 ± 35.6	228 ± 31.5
Stage 3	157 ± 76.3	239 ± 29.9	165 ± 25.0
Stage 4	246 ± 107.1	65 ± 15.5	20 ± —
Stage 5	269 ± 105.6	27 ± —	22 ± —

Table XIV

Protein-associated energy reserves per cent total energy reserve

Experimental groups	I	II	III
Stage 1	38.0	38.5	36.5
Stage 2	57.4	39.4	47.3
Stage 3	59.5	38.4	87.7
Stage 4	70.5	—	—
Stage 5	53.0	—	—

Table XV

Protein-associated energy reserves per cent total utilizable energy

Experimental groups	I	II	III
Stage 1	19.8	19.3	19.9
Stage 2	18.8	18.0	18.5
Stage 3	15.3	16.7	16.5
Stage 4	21.3	11.7	5.0
Stage 5	19.2	7.5	6.1

Table XVI

Utilizable energy used up for the synthesis of 1 g body protein (Kcal)

Experimental groups	I	II	III
Stage 1	11.6	11.0	10.4
Stage 2	19.3	12.6	12.4
Stage 3	23.3	14.3	12.6
Stage 4	18.2	—	—
Stage 5	16.0	—	—

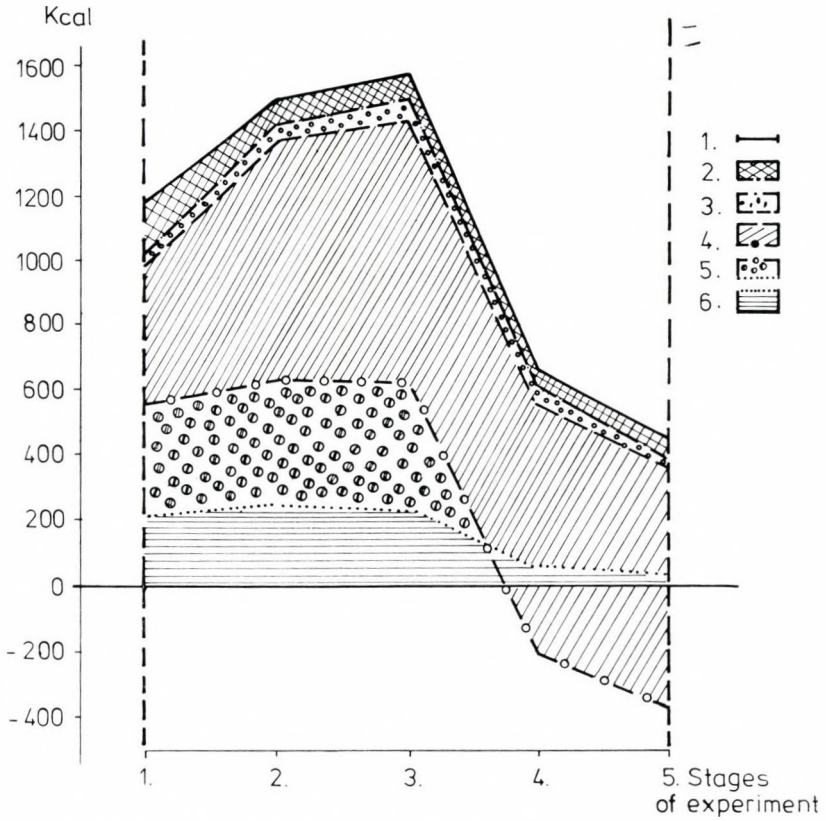

Fig. 3. Fluctuation of energy balance in group II. Explanations see Fig. 2

Discussion

According to our experimental observations, the digestibility of the milk replacer did not decrease with the progressing age of the lambs.

However, other authors have shown that the milk replacer is slightly inferior in digestibility to natural ewe's milk. JAGUS and MITCHELL (1971) gave the digestibility of ewe's milk as 98.44%.

The absolute amount of energy excreted with the urine did not increase during the experiment, but its relative proportion to total utilizable energy became practically doubled during the periods of inadequate feed supply. The energy carrying (chiefly N-containing) components of the urine represent metabolic products. With poor food supply the rate of energy metabolism will decrease in so far as the energy requirement of digestion becomes reduced, while the other life processes require practically the same amount of energy

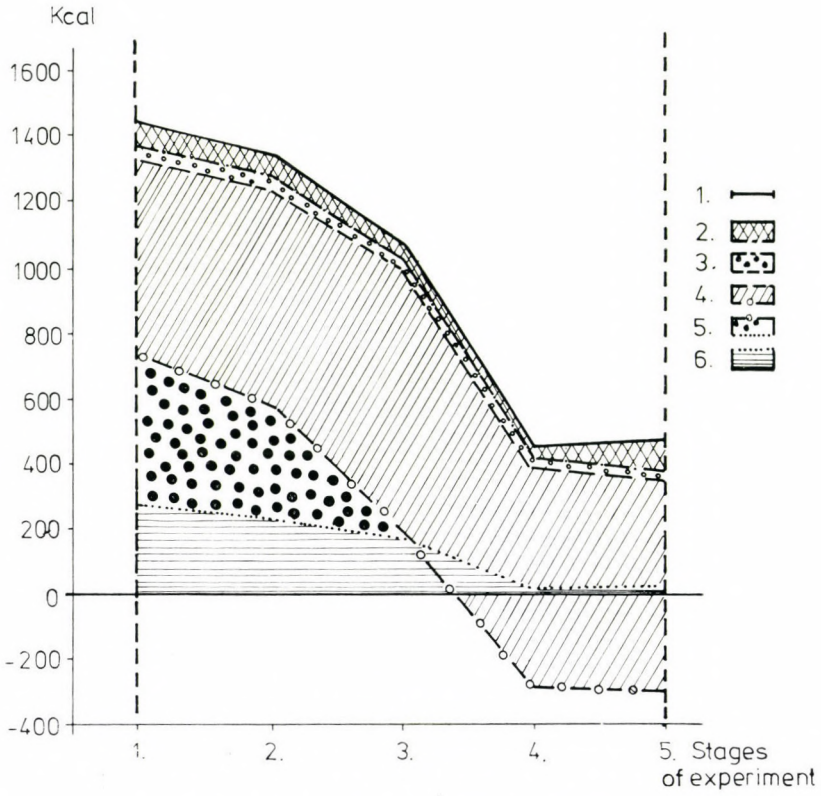

Fig. 4. Fluctuation of energy balance in group III. Explanations see Fig. 2

in normal conditions. This can explain why the urinary metabolites do not notably change in conditions of inadequate nutrition, but show a virtual increase relative to the reduced supplies of utilizable energy.

The heat loss assessed in our experimental lambs corresponded roughly to the finding of BENEDICT and RITZMANN (1931), who gave the heat production of a one-week-old ram lamb of 5.8 kg body weight as 5.0 Kcal per 1 kg body weight per hour at 18°C ambient temperature. This corresponds to a daily total of 120 Kcal. We found 114 Kcal/kg bwt/day for the lambs of group I during the first experimental period. RAFFAI and PAPP (1974) on the other hand, assessed the heat loss of a 3—5 days old lamb weighing 4 kg as 220 Kcal/day in an optimal environment.

We believe that the inequal environmental conditions bear a major, although not exclusive, responsibility for the discrepancy of the above findings.

RAFFAI and PAPP fasted the lambs for 6 h before measurements. This prolonged period of feed withdrawal may have considerably influenced the results, particularly if it is kept in mind that in natural conditions the lambs

suck the dam frequently, thus take up ewe's milk in small portions. In our experiments the lambs were fed at 3-hr intervals, took up relatively large amounts of feed on each occasion, and the metabolic measurements were performed in the interim between feedings. It should be noted that we failed to furnish an identical environmental temperature for each measurement.

The absolute value of protein-associated energy reserves tended to decrease during the experiment, but in comparison to fat-associated energy it showed a relative increase. It follows that lipid synthesis to decreased in the given conditions. Had the lambs been fed *ad libitum*, certainly the reverse would have happened, *i.e.* lipid synthesis and its energy requirement would have increased with progressing age, and protein synthesis would have decreased proportionally, as is the case in normal conditions.

The lambs utilized the dietary N-supply to the highest degree; they used up the available energy, which steadily declined in groups II and III, primarily for protein synthesis, and stored only the rest, if there was any, in the form of fat. Protein synthesis and energy storage in protein form also proceeded during the periods of negative energy balance. The energy required for protein synthesis was in all probability derived from mobilization of fat reserves, to cope with the utilization of available N. MITCHELL and JAGUS (1972) arrived at similar conclusions in lamb-feeding experiments with alfalfa hay: the animals showed a negative energy balance if the N-balance was positive, and the N-balance became negative only in the case if N was supplied with a maintenance ration.

During the first stage of our experiments the amount of energy required for the synthesis of 1 g protein corresponded to the value known from the literature (KIRCHGESSNER and MÜLLER, 1974, *etc.*), while in the later stages, in which the feed consumption was reduced, it tended to increase over the normal value.

Summary

Lambs weaned when day-old, and subsequently reared artificially on three different feeding schedules, were examined for energy and gas metabolism.

A commercial milk substitute, about 95% digestible, with 1450 Kcal total energy content per litre, was pail-fed in 20% dilution, and solid lamb starters of 3800—4100 Kcal caloric value were supplied.

The utilizable energy content was 92.5% in the milk replacer, and 78.8% in the starter.

The maintenance energy requirement of the lambs fluctuated between 109 and 76 Kcal/kg bwt/day, and it decrease with progressing age. The heat loss/h decreased correspondingly (range: 4.51—3.23 Kcal/kg bwt).

The weight gain-associated energy reserve dicreased from 720 to 0 Kcal, and the energy balance even assumed a negative value during the fourth and fifth stages of the experiment, when the energy uptake of the lambs fell below the maintenance energy requirement.

The energy reserve associated with the protein component of weight gain increased, while the lipid-associated reserve decreased, with progressing age, owing, probably, to the limited availability of feed. Protein synthesis and energy storage in protein form did not cease during the periods of negative energy balance. The lambs used up 10.4—23.3 Kcal utilizable energy for the synthesis of 1 g body protein.

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STUDIES OF DIETARY INFLUENCE ON THE HEALTHSTATE OF PREPARTURIENT COWS IN TWO DAIRY HERDS

By

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Evidence has increasingly been accumulated that cow infertility problems, particularly in high-production breeds, are often consequent upon faulty management and feeding during the dry period. Certain management-related and dietary factors may precipitate a rapidly progressive clinical illness in the dry cows through aggravation of the endogenous strain imposed upon the animals by pregnancy and calving. In certain cases illness is followed by sudden death with symptoms suggestive of hepatic coma. Such fatal outcome occurs rarely, but environmental stress during the pre-parturient and puerperal periods predisposes for various metabolic and nonspecific reproductive organ disorders, such as retention of the fetal membranes, subinvolution, retardation of sexual convalescene, and prolongation of the calving interval. In view of this, increasing importance has recently been attached to the biological control of the dry period, in order to prevent the metabolic and reproductive consequences of excessive pre-parturient stress, which tend to become more severe at higher levels of performance and with progressing age.

Material and methods

In April 1977, 15 and 12 cows in advanced state of pregnancy were selected at random from a Holstein-Friesian and a Hungarian Red Pied herd, respectively, for liver biopsy, which was then performed one, two and three weeks before calving. The cows were four to eight years old. The diet fed during the winter and early spring months consisted in both dairy farms of 25—30 kg kernel-rich maize silage, 3 kg fodder, 2.5—3 kg hay of different quality and 6—8 kg alfalfa hay; the Hungarian Red Pied cows received additionally moist and dry beet cubes and 0.5 kg molassess. The butyric acid content of the silage supplied to the Holstein-Friesian cows considerably exceeded the permissible level. The fodder ration was increased gradually

from the 14th or 15th pre-calving day on. The animals were not exercised, but were kept in a loose housing system. The dry cows were in an above-average body condition in both herds. From January to June 1977, 20 Holstein-Friesian and 22 Hungarian Red Pied cows died or had to be emergency-slaughtered after calving, on account of a sudden weight loss, listlessness, indigestion, puerperal disorders and lasting inappetence. Retention of the fetal membranes occurred in 15—25% of the cases. Puerperal intoxication and local puerperal infections were frequent. By the end of the dry period, a varying, but relatively large proportion of the cows showed depression, inappetence or fluctuating appetite. According to the records of the farm veterinarian, immediately before calving some cows in such ill health developed ketonuria, accompanied by quick respiration and symptoms indicative of a central nervous disorder (occasional tremor, extended, stiff head and neck, unusually scared expression of the eyes). Most of the cows showing such symptoms or depression were 5—8 years old.

Part of the biopsy specimens secured for light-microscopic histological examination was fixed in Schaffer's fixative embedded in paraffin, cut to sections and stained with haematoxylin and eosin. Glycogen was detected with the PAS reaction. Another part of the biopsy specimens was fixed in 10% neutral formalin, and frozen sections prepared from it were stained with Oil-red-O for the detection of lipids.

Blood samples were generally taken every other day to follow up the fluctuation of serum transaminases (SGOT, SGPT), glucose, inorganic phosporus, Ca, Mg and carotene levels.

The techniques used for the determination of the different blood parameters are listed below:

SGOT, SGPT	spectrophotometry, La Roche diagnostic kit
Blood glucose	spectrophotometry, Boehringer Biochemica-Test kit
Inorganic phosporus	spectrophotometry, Fiske-Subbarow's technique
Ca	complexometric titration
Mg	spectrophotometry, Merckotest kit
Carotene	spectrophotometry.

All spectrophotometric measurements were carried out in a MOM spectrophotometer.

Results and discussion

Histological examination revealed different degrees of hepatocellular lipid accumulation in 13 (8 Holstein-Friesian and 5 Hungarian Red Pied) of the examined 27 cows. Staining with haematoxylin and eosin showed occur-

rence of one to many cytoplasmic vacuoles in some hepatocytes; the single vacuole was as a rule very large, pushing the nucleus into one corner of the cell.

In accordance with the types of vacuolization, staining with Oil-red-O revealed fatty degeneration in the form of small or large droplets. The hepatocellular glycogen content was inversely related to the fat content. The degree of fat infiltration showed wide individual variations it was major in eight cases (mostly older cows) and minor in five. The fluctuation of the blood parameters was followed up in two animals with hepatocellular fat infiltration (Tables I and II).

Table I

Fluctuation of blood parameters between day 2 before and day 16 after calving in a 6-year-old cow exhibiting hepatocellular fatty infiltration

Date of sampling	Serum GOT	Serum GPT	Blood glucose	Inorg. P	Ca	Mg	Carotene
	mU/ml		mg/100 ml				μg/100 ml
18.4	34.5	5.0	40.0	4.5	10.5	2.8	750
26.4 (calving)	62.0	2.0	53.3	2.6	9.4	3.4	900
21.4	270.0	15.2	88.1*	6.8	—	2.7	—
22.4	248.0	12.0	50.0	9.1	8.4	2.5	82
25.4	66.0	10.8	52.3	6.8	9.3	2.2	70
27.4	175.0	8.2	36.8	5,6	9.1	2.2	110
29.4	102.0	3.8	47.3	5.2	9.7	2.0	70
2.5	62.0	2.0	33.5	7.5	9.2	2.0	100
4.5	48.0	3.5	44.4	5.4	9.5	2.5	100
6.5	44.0	2.0	40.6	6.6	9.4	1.9	145

Table II

Fluctuation of blood parameters from the second preparturient day to the tenth postparturient day in a 8-year-old cow exhibiting hepatocellular fatty infiltration

Day of sampling	Serum Got	Serum GPT	Blood glucose	Inorg. P	Ca	Mg	Carotene
	mU/ml		mg/100 ml				μg/100 ml
4.5	122	9.0	27.1	8.0	7.7	2.0	450
6.5 (calving)	245	9.0	39.6	2.4	9.0	2.4	385
9.5	256	7.4	55.6	5.1	9.2	1.4	160
11.5	182	7.9	37.2	5.0	9.4	1.4	165
13.5	122	5.8	36.7	5.6	9.7	1.9	145
16.5	34	3.8	53.2	7.1	9.7	1.8	100

Two days before calving the blood glucose level was determined as 27 and 40 mg/100 ml, SGOT as 122 and 34.5 U/ml, and SGPT as 9.0 and 5.0 U/ml, respectively. After calving, SGOT and SGPT fluctuated in the ranges 34—270 and 2—15.2 U/ml, respectively. The serum Ca and blood glucose levels were conspicuously low — near the lower physiological limit — and the inorganic P showed extreme fluctuations in both cases. The carotene level, high before calving, fell to 70—100 μg/100 ml after it, and fluctuated within this range throughout the period of study. Intermittent fluctuation of the serum transaminase levels normalized parallelly with the clinical condition of the animals as a result of daily post-parturient therapy for the period of one week.

Post-calving occurrence of "acetonaemia" or "spontaneous ketosis" in cows with above-average body fat has been reported by many authors. A subclinical form of spontaneous ketosis may already ensue during the pre-calving period. According to WOLTER (1973), cows fed high rations of maize silage are particularly liable to this disorder, for they usually put on excess fat by the end of gestation, owing to energy overdose in the pregnancy diet. Excess deposition of fat in characteristically of the abdominal region in such cases. Intake of large amounts of highly nutritive, easily digestible maize contributes to indigestion in the forestomachs. Feeding of sour maize silage may give rise to ruminal acidosis, development of which is promoted by simultaneous intake of increasing fodder but low hay rations. (ADLER et al., 1958; COPPOCK, 1974; DUNLOP, 1972; ESPINASSE, 1971; MORROW, 1976). Altered ruminal pH can adversely affect the composition of the ruminal microflora, which plays a determining role in the metabolism of carbohydrates and proteins, and can also interfere with the motility of the forestomachs (DUNLOP, 1972). These effects ultimately result in dislocation of the abomasum (COPPOCK, 1974; ESPINASSE, 1971; ROBERTSON, 1968). The chronic acidosis accounts for the absorption of toxic materials, which will sooner or later lead to liver injury, depression of hepatic enzyme activity and detoxifying capacity, and thereby to disturbances of the intermediary metabolism. Poorly preserved silage with a high butyric-acid content can easily give rise to such disorders (ADLER et al., 1958; BURIANA, 1964).

It is known that the function of the autonomic nervous system become labile during the preparturient period. Pertinent studies have revealed sympatheticotonia and increased parasympatheticotonia in 20 and 30% of parturient cows, respectively (RÜSSE, 1965). Decrease of the carbohydrate, lipid and protein reserves and simultaneous accumulation of metabolic products may give rise of acidosis (lactacidaemia) FREI, 1966). A slight sympatheticotonia is characteristic of terminal pregnancy also under physiological conditions. Approaching lactation, as well as the strain imposed upon the reproductive organs (myometrium) by parturition and subsequent involution

suddenly require increased supplies of energy. If, owing to the disturbed digestive function of the forestomachs, the energy need cannot be satisfied, a negative energy balance will ensue even on an energy-rich diet, accounting for mobilization of the lipid reserves and, sooner or later, for hepatocellular fatty infiltration.

Metabolic overload of the liver (indicated in the present cases by hepatocellular fatty infiltration, pre- and post-parturient clinical state and laboratory tests) prevents restoration of the energy balance, and may lead to some form of metabolic disease and, leads an every case a to puerperal and/or involution disorders. A good example of this is presented by the first-six-month history of the Holstein-Friesian dairy herd in 1977. A total of 272 cows had calved; of these 36 (13.2%) retained the fetal membranes, 90 (33%) had been treated several times for uterine disorders or ovarian cyst during the involution period, and 28 (10%) had required udder therapy against catarrhal or parenchymatous mastitis of varying severity. Within the first three months following upon calving, 129 cows (47.4%) either had not been on heat or failed to become pregnant. After five months 71 cows (26.0%) were still not with calf despite several treatments and inseminations. Among the cows treated for genital-tract disorders 75 (80%) among those diseased in mastitis 23 (85%) were 4—7 years old. Twenty cows (7.3%) had to be emergency-slaughtered on account of puerperal and/or metabolic disorders severe mastitis.

Many authors observed that cows liberally supplied during the dry period with maize silage and fodder rations showed a significant increase of post-calving ketosis, parturient paresis, indigestion, abomasal dislocation and endometritis incidence over those kept on liberal hay but balanced energy diet (Belyea et al., 1975; Holter et al., 1973; Kronfeld, 1971; Thomas et al., 1970; Trimberger et al., 1972). Excessive dietary supply of energy accounts for increase of the fat reserves which, however, represent a "negative surplus" by causing obesity and, ultimately, the metabolic disorder known as fat-cow syndrome (Morrow, 1976).

The cows studied by us obviously put on excess fat primarily in consequence of abundant maize silage supplies during the dry period. Obesity predisposed, above all the older cows, to parturient metabolic and reproductive organ changes sometimes leading to complications. The deleterious effect of the imbalanced diet was further increased by the faulty preservation and the high butyric-acid content of the silage. Faulty feeding depresses the adaptability of the animals to the stress imposed upon their organism by gestation and parturition. Thus the diet-induced metabolic and puerperal disorders are clearly multifactorial and as such generally irresponsive to the usual therapeutic approaches. It should also be taken into consideration that "steaming up" of the energy resources is less efficient and less economical during the

dry period than in late lactation (MOE et al., 1976). In order to reduce the incidence of diseases falling into the so-called "partus syndrome" category the maize silage ration of pre-parturient cows should be limited to about 13—14 kg daily, depending on the body condition (MORROW, 1976). Exercise by walking should be practised to prevent obesity. The fodder ration should be gradually increased from the 15th pre-calving day until term to promote adjustment of the ruminal microflora and altogether of the digestive tract for maximum energy intake during lactation.

Summary

Liver biopsies were taken one, two and three weeks before calving from a total of 27 preparturient cows belonging to a Holstein-Friesian and a Hungarian Red Pied dairy herd. During the dry period the animals were fed a diet including high maize silage and fodder, but low hay rations. The silage used in the Holstein-Friesian herd was poorly preserved and contained excessive butyric acid. Histological examination of the liver biopsies revealed hepatocellular fatty infiltration in 8 of 15 Holstein-Friesian and 5 of 12 Hungarian Red Pied cows. The cows of both herds developed an above-average body condition for lack of exercise and pasture grazing. In two animals, both clinically ill, blood analysis showed considerable fluctuation of the serum transaminases, to a lesser extent of blood glucose, carotene, Ca and P levels. Among 272 Holstein-Friesian cows which had calved during the first semester of the calendar year, 13.2% had retained the fetal membrane, 33% had uterine disease or ovarian cyst, 10% had mastitis, and 26% were still not pregnant five months later.

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PERIPARTAL BLOOD PROFILE STUDIES IN HIGH-PRODUCTION DAIRY COWS WITH SPECIAL REGARD TO REPRODUCTIVE REACTIVATION

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Regular surveys of the metabolic function of high-production dairy herds are indispensable for the maintenance of an even milk yield. Metabolic studies may be of great prognostic importance especially in the peripartal period. Transition from pregnancy to lactation is in itself a great stress, which may be further aggravated by faulty management and feeding during the dry period, and by progressing age. The peripartal energy requirement is high, and its rapid increase during the transition period can be portrayed by the comparison of pregnancy and lactation needs.

While the preparturient cow requires 18 000—20 000 Cal. daily, the lactating cow uses up at least initially, 35 000—37 000 Cal. for 20 litres, and 45 000—50 000 Cal. for 30 litres of daily yield (KOLB, 1973). Faulty feeding during the dry period disturbs the normal course of ruminal metabolism, and the surplus energy requirement is consequently derived from the organism's own fat reserves, whose decomposition account for ketogenic processes and pathological alteration of the liver function. The term "blood profile", proposed by PAYNE et al. (1970, 1974), refers to a set of blood parameters which are conclusive of metabolic function. The blood profile studies performed by us slightly differed from the metabolic profile test proposed by PAYNE et al. (1974) because we also took reproduction-biological aspects into account. The modified profile, too, makes possible, within certain limits, the prevention of the clinical expression of major metabolic disorders, on the one hand and it serves as a safe guideline for ensuring uncomplicated puerperal period and involution, as well as optimum timing of the next service.

Materials and methods

Twenty clinically healthy, preparturient Holstein-Friesian cows, 5—8 years old, were selected at random for blood profile studies during September and October 1977. Each cow had previously produced more than 7000 milk per annum, with butterfat yields ranging from 3.11 to 4.78%. From the third pre-calving week a daily ration of 3 kg dairy concentrate (soybean), 2.5 kg mixed fodder (maize, bran and barley), maize silage, and 6—7 kg alfalfa hay was fed. During the first five weeks of the dry period, 20—25 kg maize silage and 6—8 kg alfalfa hay were supplied daily. The animals were neither exercised,

Table I

Blood parameters studied and method of determination

Parameter	Abbreviation	Unit	Determination
Serum glutamateoxalic acid transaminase	SGOT	mU/ml	Spectrophotometry, MOM apparatus, La Roche diagnostic kit
Serum glutamatepyruvate transaminase	SGPT	mU/ml	Same
Lactate dehydrogenase	LDH	mU/ml	Spectrophotometry, MOM apparatus, Boehringer-Biochemica-Test kit
Glucose	G_1	mg/100 ml	Same
Urea N	BUN	mg/100 ml	Same
Cholesterol	Ch	mg/100 ml	Spectrophotometry, MOM apparatus, Boehringer-Biochemica-Test kit
Calcium	Ca	mg/100 ml	Complexometric titration
Inorganic phosphorus	P	mg/100 ml	Spectrophotometry, MOM apparatus, Fiske-Subbarow's method
Magnesium	Mg	mg/100 ml	Spectrophotometry, MOM apparatus, colour reaction with salicylaldehyde
Haematocrit value	Hc	%	Micro-method
Haemoglobin	Hb	mg/100 ml	Cyanide-haemoglobin test (KAMPEN, E. J. and ZHILJSTRA, W. G.) Vitaron haemoglobinometer

nor put to pasture. During a 8—10-day period before and after calving, blood samples were withdrawn from the jugular vein every other day, on 7—8 occasions per animal. Enzyme and glucose levels were determined on the day of sampling, the other parameters after storage of the sample at —18 °C overnight. The parameters determined by serum analysis are listed in Table I.

The mean values and standard deviations ($x \pm S$) of the blood parameters determined before and after calving, in the so-called peripartal period, are shown in Table II.

The animals designated in Table II as symptom-free were those without signs of post-parturient illness and with normal onset of lactation. Among

Table II

Pre- and post-partal values of the examined blood parameters

Serum levels of	Cows symptom free or ill.	Days 8–5	Days 4–1	Calving	Days 1–4	Days 5–8
		$\bar{x} \pm S$	$\bar{x} \pm S$	$\bar{x} \pm S$	$\bar{x} \pm S$	$\bar{x} \pm S$
GOT	s. f.	38.5±4.5	36.9±6.7	41.3±12.8	55.8±17	117.1± 83.6
	ill	35.1±4.6	37.1±4.7	95.7±72.6	197.2±153.2	213.3±116.6
GPT	s. f.	6.0±2.1	4.9±1.5	5.0±0.9	5.2± 1.8	5.9± 2.9
	ill	6.2±1.6	7.2±3.9	8.7±3.1	17.3± 13.1	19.5± 11.6
LDH	s. f.	399±201	477±244	515±268	846±355	988±405
	ill	543±276	494±243	784±684	1314±767	1178±335
Cholesterol	s. f.	93.0±9.3	89.1±17.9	82.1±17.8	90.3± 13.2	89.6± 18.3
	ill	97.1±8.5	90.5±10.7	103.6±23.1	90.0± 12.0	89.5± 32.2
Glucose	s. f.	40±4.5	41.1±3.4	47.8±2.7	35.2± 10.1	20.9± 10.1
	ill	42.6±3.2	41.8±1.9	65.8±11.3	38.8± 10.7	43.1± 12.3
Ca	s. f.	9.5±0.6	9.7±0.5	8.9±0.7	8.7± 0.9	9.1± 0.5
	ill	9.6±0.3	9.3±0.3	7.2±1.9	8.0± 1.5	8.6± 0.9
Inorg. P	s. f.	5.8±1.3	6.0±1.1	4.5±0.8	5.4± 1.1	5.4± 1.2
	ill	5.3±1.1	6.0±0.5	2.4±0.7	4.7± 1.8	4.9± 1.0
Mg	s. f.	2.74±0.35	2.8±0.24	2.96±0.42	2.96± 0.5	2.32± 0.38
	ill	2.56±0.2	2.55±0.33	3.7±0.41	2.73± 0.79	242± 0.79
BUN	s. f.	21.7±7.8	18.7±4.3	17.3±5.1	17.7± 6	18.1± 6.3
	ill	23.9±7.6	26.3±5.4	16.8±4.2	21.1± 7.1	16.4± 4.5
Haemoglobin	s. f.	11.9±1.4	11.8±1.1	13.3±0.8	12.2± 1.3	12.1± 1.0
	ill	12.5±1.6	11.9±2.1	13.2±2.3	13.0± 1.3	11.7± 1.0
Haematocrit	s.f.	35±3	34±3	38±3	35± 3	34± 2
	ill	34±5	32±5	36±7	36± 4	32± 3
Ketone	s. f.	3.2±1.2	3.5±1.6	3.1±1.0	3.9± 1.9	7.8± 3.9
	ill	2.4±1.8	3.3±1.0	1.3±0.8	3.6± 0.8	4.0± 1.6

Sympton-free n = 14; diseased n = 6

those classified as "ill", two had been recumbent since calving and four from the next day; all were listless and reluctant to rise spontaneously or on instigation. Two sick animals recovered after the usual treatment (glucose-calcium infusion plus Deltacortril injection on two occasions, at a 24-hr interval); the remaining four animals scarcely responded to therapy, and showed alternately improvement and loss of condition and appetite, until they were finally emergency-slaughtered several weeks after calving. At postmortem examina-

Table III

Peripartal normal mean values of the parameters as assessed in 10 healthy cows

Serum parameters	Prepartal days		Calving	Postpartal days	
	8— —5	—4 —1		1—4	5—8
SGOT	37.3 ± 6.6	40.6 ± 6.2	39.0 ± 2.3	62.4 ± 31.4	58.6 ± 18.6
SGPT	5.6 ± 0.6	5.7 ± 1.3	5.3 ± 1.0	6.3 ± 1.4	6.8 ± 1.3
LDH	641 ± 234	903 ± 494	1086 ± 234	1113 ± 312	1121 ± 222
Cholesterol	100 ± 23.4	117 ± 29.1	100.4±22.6	108.7± 19.5	110.3± 17.4
Glucose	54.0± 13.5	50.7 ± 16.7	45.5±14.0	51.3± 17.1	41.2± 12.3
Ca	10.0± 0.3	9.9 ± 0.3	9.2 ± 1.1	9.3 ± 1.2	10.2± 0.5
Inorg. P	6.4 ± 0.8	5.6 ± 1.1	4.9 ± 1.8	5.4 ± 1.2	5.5± 0.9
Mg	2.4 ± 0.4	2.5 ± 0.4	2.7 ± 0.3	2.7 ± 0.3	2.7± 0.4
Urea—N (BUN)	18.8 ± 3.0	17.8 ± 3.1	17.3 ± 3.8	17.5 ± 4.0	17.7± 4.8
Hb	11.7 ± 0.8	11.7 ± 1.2	11.8 ± 1.6	11.8 ± 0.8	11.6± 1.1
Haematocrit	34.0 ± 2.0	34.0 ± 4.0	35 ± 4	34 ± 2	34 ± 3
Ketone	1.8 ± 0.9	1.8 ± 1.3	2.0 ± 1.1	1.8 ± 0.7	2.0 ± 0.8

Table IIIa

Significance of the differences between the blood parameters of 20 examined and healthy cows

	Prepartal days s. f.: ill:	Calving day, s. f.: ill:	Postpartal days 1—4, s. f.: ill:	Postpartal days 5—8, s. f.: ill:
GOT	— —	— ill: $P<0.05$	— ill: $P<0.05$	s. f.: $P<0.05$ ill: $P<0.001$
GPT	—	ill: $P<0.01$	ill: $P<0.01$	ill: $P<0.01$
Cholesterol	s. f.: $P<0.01$	s. f.: $P<0.05$	s. f.: $P<0.05$	s. f.: $P<0.05$
Glucose	s. f.: $P<0.05$	— ill: $P<0.01$	s. f.: $P<0.01$ —	s. f.: $P<0.01$ —
Ca	ill: $P<0.01$	— ill: $P<0.05$	— —	s. f.: $P<0.001$ ill: $P<0.001$
Inorg. P	—	ill: $P<0.01$	—	—
Mg	—	ill: $P<0.001$	—	—
BUN	ill: $P<0.01$	—	—	—
Ketone ill: $P<0.05$	a. f.: $P<0.05$ ill: $P<0.05$	s. f.: $P<0.05$ —	s. f.: $P<0.01$ ill: $P<0.001$	s. f.: $P<0.001$ ill: $P<0.01$

tion fatty degeneration of the liver or nephritis was established. In 10 cases fluctuations of the plasma progesterone level were followed up for 3—5 postparturient weeks by radioimmunoassay.

The normal blood parameters assessed in 10 healthy Holstein-Friesian cows during the peripartal period are presented in Table III for comparison. These cows were 5—8 years old, and had a mean annual milk yield of about 6500 l. They were out on pasture in all seasons except winter. No disease of the "partus syndrome" category had occurred in the herd. The herd average for the calving interval was 380 days.

The differences found between the blood parameters of the 20 experimental and the 10 "normal" cows are shown in Table IIIa.

In Table IV are presented the parameter values described in the literature as normal average for the peripartal period.

Table IV

Normal mean peripartal blood parameters assessed by different authors

Parameter	Prepartal days		Calving	Postpartal days			Authors
	8—6	3—1		1—3	4—6	6—8	
SGOT, mU/ml	36.8 ±10.4	38.1 ±9.8	39.7 ±12.5	43.3 ±12.9	39.6 ±12.0	38.9 ±11.8	BOSTEDT, H. (1974)
SGPT, mU/ml	7.5 ±3.3	7.1 ±3.5	7.1 ±3.7	5.8 ±3.3	5.9 ±3.5	5.5 ±3.0	
LDH, mU/ml	761	783	812	803		811	SEIDEL et al. (1973)
Cholesterol, mg/100 ml	129.5 ±62.5			129.3 ±67.6		147.7 ±60.9	
Glucose, gm/100 ml	49.8 ±17			61.5 ±15.8		51.0 ±11.6	SOMMER, H. (1970)
Ca, mg/100 ml	9.74	9.98	8.27	7.86		9.51	HEVETT, CHR.
Inorganic P, mg/100 ml	6.32	5.82	4.42	4.46		5.33	(1974)
Mg, mg/100 ml	2.10	2.29	2.53	2.54		2.10	
BUN, mg/100 ml	17.3						
Haemoglobin, g/100 ml	11.2 in the peripartal period						HEVETT, CHR. (1974)
Haematocrit, %	28—32						HORVÁTH, Z. (1975)
Ketone, mg/100 ml	4—7						KARSAI, F. (1975)

Results

The SGOT level of symptom-free cows rose to 117.1 ± 83.6 mU/ml by 5—8 days post partum, while that of the sick animals was as high as 95.7 ± 72.6 mU/ml already at calving, and rose significantly ($P < 0.001$) during the subsequent days. According to BOOTS et al. (1969) and LOTTHAMMER et al. (1971), the SGOT activity tends to decrease in cows with age and with progressing gestation (from 42.9 mU/ml to 30.5 mU/ml), but rises post partum. The mean SGPT activity (8.7 ± 3.1) established in the sick animals at calving increased more than twofold ($P < 0.01$) by the eigth post-partum day. In physiological conditions the SGPT activity rises before and after calving, occasionally up to the highest physiological limit. The LDH level tended to increase post partum in both healthy and sick animals, especially in the latter. PRANGE (1964) observed that the LDH enzyme activity was generally not notably altered by gestation, but it tended to rise during the puerperal period, owing probably to the physical strain of calving and the onset of involution. Cellular injury in the organs containing highest levels of the enzyme (liver, heart, skeletal muscles) accounts for an index-like rise of activity in the serum. The primary site of cholesterol synthesis is in the liver. Cholesterol is vitally important as basic source of steroid genesis, gonadal and adrenal hormones, and vitamin D_3. The cholesterol level usually tends to decrease during the last week of pregnancy until calving, and falls to a minimum in the early puerperal period (LOTTHAMMER et al., 1971; SOMMER, 1970; TRÄNKNER, 1971). SOMMER (1970) established that alterations of the serum cholesterol level always signify disturbance by metabolic changes or liver injury of its synthesis, release or utilization. Drop of the cholesterol level is generally consequent upon liver injury, hyperthyroidism, or faulty feeding. In the present study significantly ($P < 0.01$; $P < 0.05$) low cholesterol levels were found during the immediate pre- and post-calving days, above all in the symptom-free cows. Other authors (HUHN and LUPKE, 1962; SOMMER, 1970; LOTTHAMMER et al., 1971) reported a continuous decrease in serum glucose from seven months of pregnancy, followed by an abrupt rise at calving. We found a mean level of 20.9 ± 10.1 mg/100 ml not earlier than by 5—8 days post partum, and only in the symptom-free cows. The difference between the symptom-free animals and the healthy ones used as control was established in terms of significance as $P < 0.01$, $P < 0.01$ and $P < 0.001$, respectively, at 5—8 days before, and 1—4 and 5—8 days after calving. (Unlike the sick cows, the healthy ones received no glucose therapy). The sick cows had lower serum Ca and inorganic P levels before, during and after calving than symptom-free ones had post partum. The inorganic P level was especially low during calving, 2.4 ± 0.7 mg/100 ml. The serum Ca and inorganic P levels of healthy cows differed significantly from those assessed in the sick cows 1—4 days before, during, and 5—8 days after calving

($P < 0.01$; $P < 0.001$, respectively). The inorganic P levels differed at calving at the $P < 0.01$ level of significance. According to literary data the inorganic P level does not change significantly during gestation (EKMAN et al., 1970), but shows a definite decline at calving, and rises gradually post partum, until it reaches a peak value at about 4 months after calving (HEVETT, 1974). In physiological conditions, the peripartal drop of serum Ca and inorganic P was generally assessed as 1—2 mg/100 ml. A greater decrease can probably also occur with progressing age and number of lactations (JÖRGENSEN, 1974). In the present study, both the symptom-free and sick cows had elevated serum Mg levels (2.96 ± 0.42 and 3.71 ± 0.41, respectively), and the sick ones showed a significant ($P < 0.001$) increase of it at calving. The blood urea nitrogen (BUN) rose markedly ($P < 0.01$) in the sick cows and less markedly in the symptom-free ones during the prepartal days. According to literary data, blood urea nitrogen drops physiologically during gestation, reaching a minimum in the peripartal period, after which it tends to increase slowly until 2 months after calving (HEVETT, 1974; BLOWEY et al., 1973). The haemoglobin concentration was also elevated in both sick and symptom-free animals; the mean concentration, assessed in the sick ones at calving as 13.2 ± 2.3 g%, did not change for 1 to 4 subsequent days. The haematocrit values, too, were generally elevated. It is known that in physiological conditions the haemoglobin concentration generally tends to decrease during terminal pregnancy, tends to rise after calving, and then tends to decline again until about 2—4 months after calving (HEVETT, 1974). The blood ketone level showed post partum a significant increase over normal (Table III) in all 20 cows studied.

Fluctuations of the plasma progesterone level were determined in blood samples taken from 10 cows usually every third day during a 21—35-day period post partum, to follow up ovarian function and the revival of the reproductive cycle. The progesterone levels determined by radioummunoassay are shown in Table V.

Discussion

The blood profile studies clearly indicate that those cows which became diseased post partum had shown a notable elevation of the SGOT and SGPT activities already at calving, and on the consecutive days as well. The sick cows also had significantly depressed serum-Ca levels before, during and, specially after, calving. A not less significant decrease was observed post partum in the symptom-free cows. At calving, the sick cows had significantly lower serum P levels than the symptom-free ones; the difference was significant relative to healthy cows as well. Blood urea nitrogen was elevated during the preparturient days in all 20 cows, even significantly so in the ones which became diseased post partum. It is known from literary data that protein uptake may in-

Table V

Fluctuation of progesterone levels (ng/ml) in 10 healthy cows for 21—35 days post partum

Day of sampling	Cow No.			Day of sampling	No. of animal						
	626	354	29		794	764	87	183	520	747	317
11.9.	2.21	—	—		P. 31.10	P. 26.10	P. 27.10	P. 24.10	P. 26.10	P. 30.10	P. 31.10
12.9.	2.78	6.55	3.62	2.11.	0.21	<0.20	<0.20	<0.20	<0.20	<0.20	<0.20
14.9.	2.52	3.87	2.41	4.11.	0.66	0.23	<0.20	<0.20	<0.20	<0.20	<0.20
16.9.	P. 0.67	3.51	P.<0.20	9.11.	<0.20	<0.20	<0.20	<0.20	<0.20	<0.20	<0.20
19.9.	1.11	3.20	<0.20	12.11.	0.31	<0.20	0.22	<.020	<0.20	<.020	<0.20
21.9.	2.11	P. 0.78	0.21	15.11.	0.23	<0.20	0.54	<0.20	<0.20	<0.20	<0.20
23.9.	1.74	0.65	<0.20	18.11.	0.40	0.22	1.32	<0.20	<0.20	<0.20	<0.20
27.9.	1.19	<0.20	<0.20	21.11.	<0.20	0.29	3.23	<0.20	0.63	<0.20	<0.20
29.9.	0.86	0.31	<0.20	24.11.	<0.20	<0.20	4.45	<0.20	—	<0.20	<0.20
3.10.	0.88	0.27	0.42	28.11.	<0.20	<0.20	4.61	<0.20	0.78	1.98	<0.20
6.10.	1.72	<0.20	<0.20								
10.10.	—	0.28	—								
13.10.	—	0.54	—								
Service period (days)	230	220	225		195	80	60	190	120	190	140

P, progesterone level found on calving day, or day of partus

fluence the blood urea nitrogen level (PRESTON et al., 1965; PREVITT et al., 1971); animals on high-protein diets generally show elevated UN and total protein values (RUSSEL, 1974). Rise of the SGOT level may signify excessive dietary protein and energy supplies (WETTKE, 1971) accounting for overloading and, consequently, injury of the liver. Cows so affected show a marked liability to post-partum involution disorders, endometritis and anoestrus (LOTTHAMMER et al., 1971). A positive correlation seems to exist between these disorders and drop of the blood glucose level, which was significantly low in the symptom-free cases during the post-partal days. A similarly close correlation has been established between the carbohydrate metabolism and the serum inorganic P level (SIMESEN, 1970), which tends to fall during increased carbohydrate utilization, and usually rises on starvation. Influence of the diet composition on the haemoglobin concentration has also been implied (GREIG and BOYNE, 1956). Drop of the haemoglobin concentration after seven months of lactation was attributed to reduction of the animals to a lower plane of nutrition during the dry period, on the grounds that dry cows maintained at a high plane of nutrition exhibited higher haemoglobin values (HEVETT, 1974).

It is known that with an uncomplicated puerperal course, the first ovulation may ensue as early as 15 days post partum, without heat symptoms, and a second one, usually also silent, follows at about 30—32 days. Heat symptoms associate with ovulation as a rule only after a third process of follicle maturation 18—20 days later (on days 45—50 post partum). This was obviously not the case with the 10 cows studied for progesterone level, for throughout the sampling period the latter was either as low as the minimal level shown by ovariectomized cows, or scarcely higher, indicating delay of ovarian reactivation, disturbance of steroid hormone genesis, and consequent prolongation of the involution process.

The reproduction-biological data of the diseased and symptom-free cows are shown in Table VI.

Table VI

Post partum reproduction-biological events among the diseased and symptom-free cows

Cows	n	Emergency-slaughtered	Recovered	Non pregnant on day post partum	Pregnant. Days from calving to conception
Diseased	6	4	2	1 225	1 140
Symptom-free	14	1	—	8 190—240	5 60—120
Totals'	20	5	—	9	6

It is concluded from the experimental observations that both the sick and symptom-free cows showed a minor to major deviation from normal of all examined blood parameters except SGPT and inorganic P during the peripartal period. Sexual convalescence took a markedly prolonged course in the apparently healthy cows, too. Among the examined 20 cows only 6 (30%) became pregnant between days 60 and 140 post partum, and between days 190 and 240 nine cows (45%) were still not in calf. Five cows of the group studied (25%) had to be emergency-slaughtered after a shorter to longer time post partum. The infertile cows had been treated for endometritis or cystic ovary, but failed to conceive on repeated insemination. The underlying mechanism of the reproductive disturbance, which accounted for a considerable prolongation of "open days" and service period, had been in all probability a latent disorder of the protein and energy metabolism, ultimately resulting in injury of the hepatic enzyme activity and cholesterol synthesis.

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Summary

Twenty Holstein-Friesian cows of high milk yield, all older than 5 years, were examined ford blood parameters of metabolic and reproduction-biological significance in the peripartal period covering 8—10 days partly before, partly after calving. Six cows of the group became diseased — showed recumbency — post partum, 14 remained symptom-free. The sick animals showed a considerable elevation of SGOT and SGPT as well as serum LDH activities, while their serum Ca and, especially, inorganic P levels dropped at calving relative to normal. During the preparturient days the blood urea nitrogen level of the sick cows increased by about 30—40%, that of the symptom-free cows by about 10—20% over the normal average. The cholesterol level was conspicuously low in each case, and even the symptom-free animals showed a minor to major shift of the examined blood parameters, with the exception of SGPT and inorganic P.

The plasma progesterone levels followed up for 21—35 days post partum corresponded in most cases to the minimum concentration found in ovariectomized cows. Among the 20 cows studied 6 became pregnant between days 60 and 140 post partum, and 9 were still "open" between days 190 and 240; five animals had to be emergency-slaughtered after a shorter or longer time post partum. The infertile cows failed to conceive after simed treatment and repeated inseminations; the reproductive disturbance became expressed in a considerable increase of "open" days.

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TRANSMISSION OF ZEARALENONE AND ITS METABOLITE INTO RUMINANT MILK

By

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Zearalenone [2,4-dihydroxy-6-/10-hydroxy-6-oxo-trans-1-undecenyl/-β-resorcylic acid--μ-lactone] *i.e.* F-2 toxin (Fig. 1) is an estrogenic metabolite of several species of the fungus genus *Fusarium* (PATHRE and MIROCHA, 1976). In particular, SNYDER and HANSEN are known as having many *Fusarium roseum* isolates which are strong producers of zearalenone in culture (BOLLINGER and TAMM, 1972; STIPANOVIC and SCHROEDER, 1975; PATHRE and MIROCHA, 1976; HAGLER et al., 1979). Zearalenone is a fairly common contaminant of cereal feedstuffs in levels which can cause the well-documented hyperestrogenic syndrome in swine (PATHRE and MIROCHA, 1976).

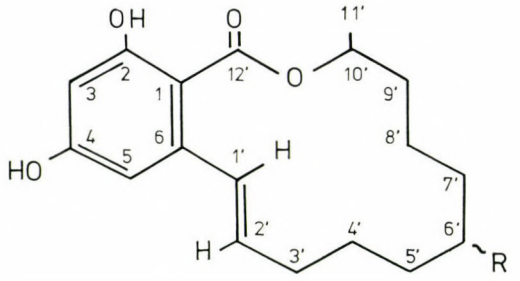
The effect of low levels, or chronic exposure to low dietary levels, on male and female animals is poorly understood, though VÁNYI and SZAILER (1974) examined the effect of zearalenone in low levels on monolayer tissue cultures of testicular tissue from several species. They found swine the most sensitive and rooster the least sensitive species. These results correlate well with relative sensitivities seen in nature and in other experiments where zearalenone was fed in the diet. In female animals sows were infertile, and those already pregnant showed tendency to abort or reabsorb fetuses (CHANG, 1978). Some piglets were born with deformities which indicated possible teratogenic activity for zearalenone.

Zearalenol [2,4 dihydroxy-6-/6,10-dihydroxy-trans-1-undecenyl/-β-resorcylic acid-μ-lactone] was discovered as a naturally occurring metabolite of *F. roseum* 'Gibbosum' and 'Graminearum' by STIPANOVIC and SCHROEDER (1975) (Fig. 1). HAGLER et al. (1979) found only alpha-zearalenol in cultures of *F. roseum* 'Gibbosum'. Alpha-zearalenol has been reported to be about three times as estrogenic in the uterotropic bioassay when fed in the ration of castrated female mice (PETERS, 1972) and about four times as estrogenic as zearalenone when applied in acetone to the shaved skin of weanling female rats (HAGLER, et al., 1979). Beta-zearalenol, not found in cultures of *F. roseum* 'Gibbosum', was produced chemically and was found to be about equal to zearalenone in activity in the rat skin test, but less acitve than zearalenone in the mouse-feeding test (PETERS, 1972; HAGLER et al., 1979). Beta-zearalenol has been found as the major metabolic product of zearalenone produced by swine, rats, the yeast *Candida tropicalis*, cows, and sheep (PALYUSIK et al., 1980a; PALYUSIK et al., 1980b; PATHRE et al., 1978).

Zearalenone intubated into or fed to swine in rations is partially converted metabolically to beta-zearalenol primarily and small amounts of alpha-zearalenol and these metabolic products have been found in both urine and milk (PATHRE et al., 1978; PALYUSIK et al., 1980a). This pattern of transformation is also observed in *C. tropicalis* (PALYUSIK et al., 1980b). It is striking that the diastereomer produced by these other organisms from zearalenone is different from that produced in zearalenone-producing cultures of *F. roseum* 'Gibbosum'.

The other naturally occurring metabolites of *Fusarium*, too, are closely related to zearalenone (Fig. 2).

As in the case of aflatoxin there is concern that zearalenone and zearalenol might enter the human diet directly in cereal products or, more subtly, in the form of residues in animal tissue or animal products such as milk or other dairy products. PALYUSIK et al. (1980a) have demonstrated that zearalenone and in particular zearalenol are transmitted into the milk of sows which have been fed zearalenone in their ration and that these compounds are transmitted into the milk in amounts which can cause hyperestrogenic syndrome in the suckling piglets.



zearalenone R / O

alpha-zearalenol < H / OH

beta-zearalenol < OH / H

Fig. 1. Zearalenone and zearalenol[a].
a, Alpha-zearalenol is a metabolite of *Fusarium roseum* while beta-zearalenol and small amounts of alpha-zearalenol are transformation products of zearalenone by *Candida tropicalis* and swine

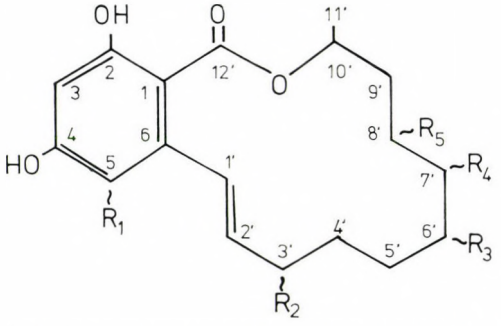


Fig. 2. Naturally-occurring derivatives of zearalenone[a].
a, Zearalenone and alpha-zearalenol from cultures of *Fusarium* have estrogenic activity. Beta-zearalenol has estrogenic activity also but has not been found in cultures of *Fusarium*. It is a product of metabolism of zearalenone by *Candida*, and an urinary metabolite of swine and rats

	R_1	R_2	R_3	R_4	R_5
Zearalenol	H	H_2	OH	H_2	H_2
Zearalenone	H	H_2	O	H_2	H_2
8'-hydroxyzearalenone	H	H_2	O	H_2	OH
3'-hydroxyzearalenone	H	OH	O	H_2	H_2
6',8'-dihydroxyzearalene	H	H_2	OH	H_2	OH
7'-dehydrozearalenone	H	H_2	O	H	H
5-formylzearalenone	CHO	H_2	O	H_2	H_2

Whether zearalenone or zearalenol like aflatoxins could be transmitted into milk in ruminants was unknown until now. We report in the present paper that zearalenone and beta-zearalenol are transmitted into cow and

sheep milk. Beta-zearalenol and zearalenone were also identified in the urine of the ewe treated with zearalenone. Under these conditions, only traces of zearalenone and beta-zearalenol were found in the cow milk. These compounds were present in the sheep milk at 0.001—0.002 ppm in some extracts and female lamb receiving the ewe's milk developed hyperestrogenism.

We also describe a very sensitive and specific method of analysis of milk for these compounds.

Materials and methods

Five-gram samples of crystalline zearalenone were mixed thoroughly with about three litres of ground feed and fed to an approximately 450 kg milk cow which had consumed all feed and toxin within 10 min.

Zearalenone (1.8 g) was administered into the esophagus of a lactating ewe in 20 ml of 96% ethanol. This was followed by 60 ml of water in 20 ml doses. The ewe had a 1-week-old female lamb which had received all the milk not taken for extraction. The lamb and the ewe were observed periodically for signs of hyperestrogenism.

The cow was milked by machine morning and evening and one litre of milk was taken as a sample from each milking for 5 days and from the morning milking of the 6th day. Of each sample, 500 ml was extracted and the remainder was frozen at —10 °C. The first sample, only 250 ml, was taken just before administration of the toxin. All was extracted (Table I).

Table I

Transmission of zearalenone and beta-zeralenol into bovine milk[a]

Sample	Time (hr)	Sample volume (ml)	Zearalenone	Zearalenol *(beta)*
1	0	250	—	—
2	12	500	—	—
3	24	500	—	—
4	36	500	—	—
5	48	500	—	—
6	60	500	—	—
7	72	500	—	—
8	84	500	—	—
9	96	500	+	—
10	108	500	+	+
11	120	500	+	+

[a] Analysis by reverse-phase HPLC.

The ewe was milked by hand once, each day in the morning. Sample sizes varied (Table II) and were taken over five days. Urine samples were collected from the ewe once each day and these also varied in volume (Table III).

The extraction producere for milk and urine is illustrated in Table IV. Volumes of solvents and reagents were adjusted on a proportional basis for sample volumes other than 500 ml.

Beta-glucuronidase, 68.000 Fishman Units (Calbiochem, San Diego CA, USA), was dissolved in 100 ml of destilled water, the solution stored at 5 °C and used as needed over an eleven-day period. Extracts of milk or urine samples were adjusted to pH 4.5—5 with glacial acetic acid and 2.0 ml of enzyme preparation (1360 Fishman Units) were added to extract. Incubation was for two hr at 37 °C.

Pre-coated Kieselgel 60 plates (not activated) with layer thickness of 0.25 mm (E. Merck, Darmstadt, FRG) were used for thin-layer chromatography (TLC). The solvent system was $CHCl_3 : CH_3OH$ (95 : 5, v/v). Plates were examined under longwave UV light (360 nm) and then sprayed according

Table II

Transmission of zearalenone and beta-zearalenol into sehep milk[a]

Sample	Time (hr)	Sample volume (ml)	Zearalenone	Zearalenol *(beta)*
0	0	25	—	—
1	24	200	+	+
2	48	200	+	+
3	72	200	+	±
4	96	375	—	—
5	120	350	—	—

[a] Analysis by reverse-phase HPLC.

Table III

Transnission of zearalenone and beta-zearalenol into sheep urine[a]

Sample[b]	Time (hr)	Sample volume (ml)	Zearalenone	Zearalenol *(beta)*
0	0	100	—	—
1	24	45	+	+
2	48	65	+	+
3	72	100	+	+

[a] Analysis by reverse-phase HPLC; [b] Samples were not taken after 42 hr.

Table IV. Milk extraction procedure
Defatting step was omitted beginning with cow milk sample 6 with no problems for reverse-phase HPLC of milk extracts. Extraction of urine was carried out in the same fashion except that the pH was adjusted to 4.5—5.0 at the beginning

0.5 L Milk
+
0.5 L Acetone
↓ 1. Stirred 1 hr
↓ 2. Filtered quantitatively (No. 2 paper)
↓ 3. Precipitate discarded
Supernatant
↓ 1. Acetone removed *in vacuo* at 70 °C
↓ 2. pH adjusted to 4.5—5 with glacial acetic acid
↓ 3. Extract incubated with *beta*-glucuronidase 2 hr at 37 °C
Hydrolyzed extract
(*ca.* 225 ml)
↓ 1. Equal volume of saturated $(NH_4)_2SO_4$ added
↓ 2. Extracted with 300, 200, then 100 ml ethyl acetate
Acetonitrile extract
↓ 1. Defatted with 100, then 50 ml hexane
↓ 2. Combined hexane extracts extracted with 50 ml acetonitrile-hexane discarded
↓ 3. Acetonitrile removed *in vacuo* at 70 °C
Residue
↓ 1. Transferred quantitatively with acetone, with filtering, to a small vial.
↓ 2. Acetone evaporated at 70 °C under an N_2 stream
Analysis
1. Residue dissolved in 0.5 ml acetone and aliquots taken as needed

to the method of SARUDI (1974). Internal standards of zearalenone and zearalenol were included for all extracts.

Ten-microlitre samples of 3.0 ml total volume of extract, then 5 μl of 0.5 ml total volume of extract of milk and urine, were analyzed by TLC.

High-performance liquid chromatography HPLC was accomplished in a Packard Model 8200 instrument (Packard-Becker B. V., Delft, Netherlands) fitted with a C_{18} reverse-phase analytical column (Partisil PXS 10/25 ODS, Whatman Inc., Clifton, N. J., USA) and equipped with an UV detector, which was operated at 254 nm. Peaks were integrated using a VIDAR autolab 6300 digital integrator (Mountain View, CA, USA). Components of extracts were resolved with a solvent system of 40% methanol. Flow rate was 1.7——1.8 ml min^{-1}. Volumes of 10 or 14 μl were injected.

The syringe used for HPLC was an SGE (Scientific Glass Engineering Pty, LTD, North Melbourne, Australia) high-pressure syringe. It was crucial to ensure that the syringe was clean.

Ten mg of pure zearalenone, dissolved in acetone, was added to control milk (500 ml) from another cow in the same herd. Zearalenol was added at a similar rate and it was assumed that the efficiency of recovery was equal for these compounds. Analysis and quantification by HPLC and comparison to amounts of each equal to those added to the milk gave the percentage recovery.

The ewe and the lamb were isolated from the other livestock and at night were isolated from each other. This allowed collection of samples of

adequate volume and ensured that the lamb received all the milk not used for analysis. The lamb served then as a bioassay for transmission of zearalenone and zearalenol into the ewe's milk.

Results

Analysis of bovine milk extracts by TLC revealed a bright light-blue fluorescent spot with an R_f value just lower and overlapping with that of zearalenol. The component interfered with analysis by UV light. But this component did not react with Sarudi's reagents. All bovine milk samples were negative by analysis with TLC, but samples no. 10 and 11 showed low levels, traces of zearalenone and beta-zearalenol on analysis by HPLC (Table I).

Sheep milk and urine yielded extracts which were positive for zearalenone and beta-zearalenol in both TLC and HPLC analyses (Tables II and III). These compounds were present at 0.001—0.002 ppm in the milk and at higher levels 0.020—0.050 ppm in the urine.

Resolution of zearalenone and the diastereomeric zearalenols is shown in Figure 3. Quantitative standards examined by HPLC revealed nearly equimolar absorbances for zearalenone and alpha- and beta-zearalenol at 254 nm (not shown). Therefore, the diastereomeric mixture of zearalenol obtained by reduction of zearalenone with $NaBH_4$ (Sarudi 1976) consisted of 60—65% beta-zearelenol and 34—40% alpha-zearalenol.

Recovery of zearalenone from spiked control milk was 71%.

Within 10 days after administration of zearalenone to the ewe, the lamb showed signs of hyperestrogenic syndrome.

Discussion

In HPLC analysis no components interfered with analysis of either defatted or non-defatted extracts. In fact, the peaks for zearalenone and alpha- and beta-zearalenol were the only components seen in the chromatograms of extracts even at the most sensitive attenuation used.

Sensitivity of the assay when background was not too high or too complex, as it was in the case of the sheep urine extracts, was reliable to 50 ppm of zearalenol or zearalenone in the final extract when 10 μl volumes were injected or to traces in the milk itself. Urine samples were always too complex to interpret the chromatograms with good accuracy while the chromatograms of the milk samples never were. Faecal contamination was common in urine samples while milk was never contaminated with feces.

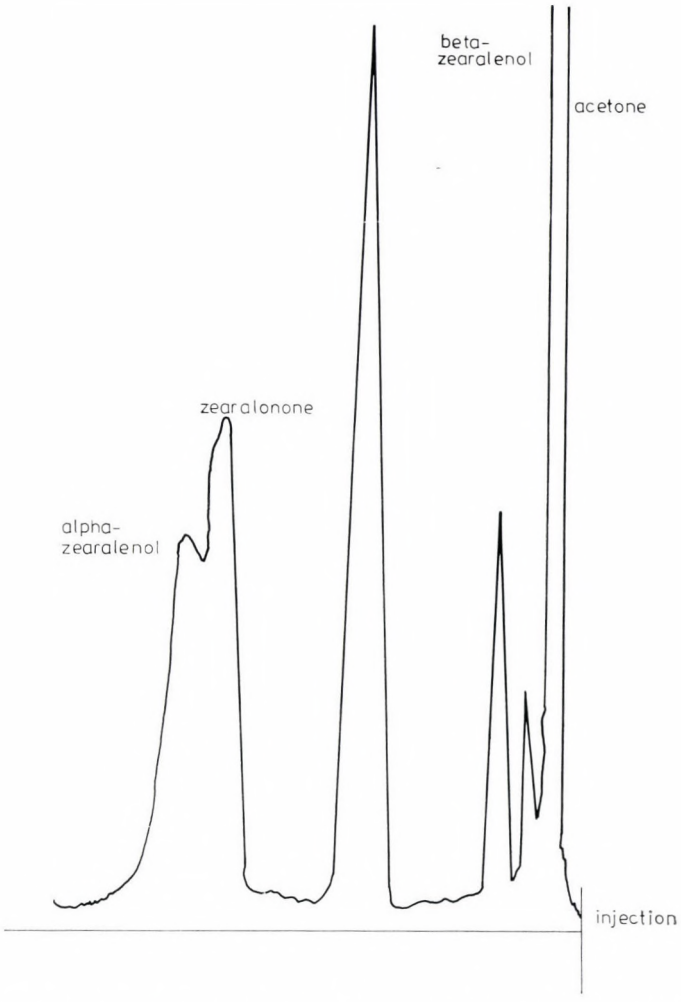


Fig. 3. C_{18} reverse-phase HPLC of control cow milk, spiked 20 ppm zearalenone and an unknown quantity of zearalenol added

The fact that zearalenone and beta-zearalenol are transmitted into milk in relatively low levels compared to the extent of transmission in swine would seem to indicate that this transmission is less efficient in ruminants. However, the hyperestrogenism in the lamb indicates transmission in levels adequate to cause problems even when analyses revealed only 0.001—0.002 ppm in the ewe's milk.

It is clear that this method with its high rate of recovery of zearalenone and zearalenol and great sensitivity is potentially a very important method for analysis of milk for these compounds. We suggest an extension of the method to other compounds of interest.

It was anticipated that the levels of compounds transmitted would be low, so the complete extract was treated with beta-glucuronidase and no attempt was made to determine the proportions of the compounds transmitted as glucuronides and those not transmitted as glucuronides or even if glucuronides were present in the milk.

If alpha-zearalenol was present in extracts it was in amounts too small to measure by this procedure.

Summary

Five grams of crystalline zearalenone in mixed feed and 1.8 grams of zearalenone in 96% ethanol were administered to a milk cow and a ewe, respectively. Extracts of milk analysed by reverse-phase HPLC revealed traces of zearalenone and beta-zearalenol in some extracts of cow milk. Zearalenone and beta-zearalenol were present in some sheep milk extracts at 0.001—0.002 ppm. Extracts of urine from the ewe also contained these compounds at levels 0.020—0.050 ppm also discernable with thin-layer chromatography within 24 hr of administration of zearalenone. The female lamb with the ewe consumed all milk not used for extraction and exibited the major sign of hyperestrogenism — an abnormally enlarged vulva — about 10 days after zearalenone had been administered to the ewe. Analytical reverse-phase HPLC is a very sensitive analytical technique for the analysis of these metabolites.

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TRANSMISSION OF ZEARALENONE AND ZEARALENOL INTO PORCINE MILK

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It is well-known that aflatoxin is metabolized in animals into a less toxic form called M_1 alfatoxin. M_1 alfatoxin appears also in the milk of lactating cows. The F-2 fusariotoxin, zearalenone [2,4-dihydroxy-6-(10-hydroxy-6-oxo-trans-1-undecenyl)-β-resorcylic acid-μ-lactone], an estrogenic toxin, is also metabolized in swine, and is eliminated in the urine as zearalenol [2,4-dihydroxy-6-(6,10-dihydroxy-trans-1-undecenyl)-β-resorcylic acid-μ-lactone] (PATHRE et al., 1978; VÁNYI and SZIGETI, 1978). However, at the time of our study, there were no data available concerning the appearance of zearalenone and its derivatives in the milk. Clinical observations (RÓZSA, 1978) indicate that the vulva of 1-2-week-old piglets becomes swollen when the lactating sow is fed with zearalenone. Since piglets of this age generally do not consume compact feed, it seems reasonable to suppose that the source of the estrogenic toxin is the ingested milk. HAGLER et al., (1980a) have shown that in the milk of cow and ewe fed zearalenone during lactation zearalenone and beta-zearalenol appears, which is approximately as toxic as zearalenone.

The purpose of this study was to show whether zearalenone unchanged, or in the form of its metabolites, appears in the milk of sows fed crystalline zearalenone after parturition.

Materials and methods

Feeding of animals with zearalenone. Mixed feed containig 40 ppm crystalline zearalenone was fed to two sows from the 8th day after parturition. During the 9-day feeding period, each sow consumed 1 g zearalenone in 25 kg feedstuff. Before starting the experiment, 2 g of crystalline zearalenone was dissolved in 1 l diethylether, and mixed thoroughly with 4 kg ground feed. After evaporation of the ether, the mixture was diluted by adding 46 kg ground feed.

The piglets were excluded from the toxic diet; they had been separated from the sow until the whole toxic feed was consumed by the sows.

The presence of the toxin in the milk was demonstrated by both biological and physico-chemical laboratory tests.

Biological test. Fifteen piglets, including 8 males and 7 females, born to two sows (9 and 6 piglets per litter) were subjected to continuous clinical observation.

Milk samples for laboratory tests were collected by milking sows in the morning. To facilitate lactation, 15 IU oxytocin (G. Richter, Budapest) was injected into the vena cava cranialis of each sow before milking. Milk samples were taken just before administration of zearalenone and, subsequently, on the 2nd, 3rd, 4th, 6th and 9th days of the toxic diet as well as on the 2nd, 5th and 8th days after the diet had been finished. The volumes of milk samples obtained were generally between 15 and 30 ml in volume, expect a single sample of 88 ml.

Extraction of the toxin from milk samples. One portion of each sample was processed as follows: 1 volume of full-cream milk was mixed with an equal volume of chloroform and 2 volumes of acetone. The precipitate was separated from the liquid phase by filter paper. Both, the precipitate and the liquid, were dried and then clarified in a separatory funnel by partitioning with an equal volume of acetonitrile and n-hexane. The acetonitrile fractions were used for testing.

The other portion of each sample was treated as follows: full-cream milk was centrifuged (at 3000 rpm for 10 min), and then the butterfat and the skimmed milk were separated by partition with acetonitrile and n-hexane. The butterfat was treated as described above while the skimmed milk dried before the partitioning. Analyses were made of the acetonitrile fractions.

The extracts were tested by several physico-chemical methods.

Physico-chemical test. For thin-layer chromatography (TLC), Merck's silica gel plates, chloroform and methanol (95 : 5 v/v) developing mixture, UV light, and the zearalenone detection method developed by SARUDI (1974) were used.

Gas-liquid chromatography (GLC): A Hewlett Packard 5710A chromatograph equipped with a hydrogen flame detector was used for GLC analyses of the milk extracts. The flow rate of nitrogen, the carrier gas, was 30 ml/min through a stainless steel column (91.44×0.318 cm) packed with 3% QF-1 on 100/120 mesh gas chrom Q. The temperature of the injector port and the detector cell was 275 °C. The dried, solvent-free extract of the milk was reacted with Tri-Sil-BT (Tierce Chemical Co. Rockford, Illinois, U.S.A.) and was injected into the column programmed from 150 to 275 °C at 8 °C/min. The standards used were trimethylsilyl (TMS) ethers of zearalenone, alpha-zearalenol and beta-zearalenol.

Gas chromatography—mass spectrometry (GC—MS): The above silylated samples were analysed by GC—MS operated in the selected ion-moni-

toring mode (SIM). A set of 16 characteristic ions of TMS ethers of zearalenone and zearalenols were monitored. The SIM profile indicated the presence of zearalenone, alpha-zearalenol and beta-zearalenol in the samples of the sow milk.

Results

The experiments have proved that when the feedstuff of lactating sows contained zearalenone 40 ppm, its metabolite (zearalenol) was appeared in the milk.

The vulvas of all the 7 female piglets, became swollen and red 4—5 days after toxin administration to their mothers, and then, gradually, the symptoms became more pronounced (Fig. 1). The uterus (Fig. 2) of one suckling piglet bled on the 5th day after termination of the 9-day feeding period and the vulva of this piglet were also enlarged. These pathological lesions indicated the presence of an estrogenic effect, but did not identify the estrogenic component in the milk.

Laboratory testing of the milk samples revealed that of the zearalenone metabolite that appeared in the milk, 82.0—86.1% was beta-zearalenol, 13.4—15.7% was alpha-zearalenol and only a negligible percentage

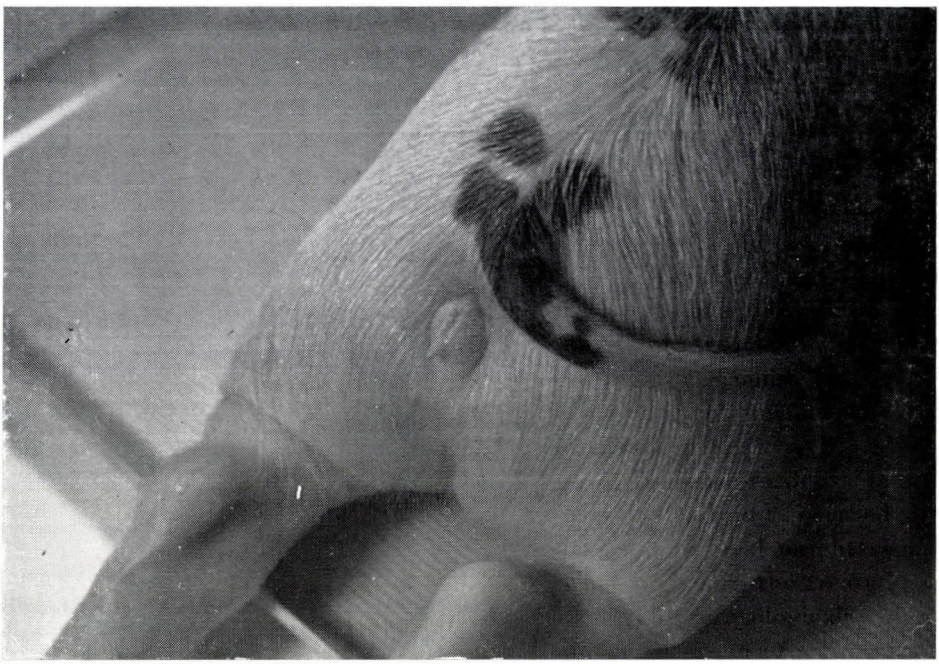

Fig. 1. The enlarged vulva of a 21-day-old sucking piglet of the sow fed with zearalenone 40 mg/kg feed continously for 9 days. The piglet was killed 5 days after feeding of the toxic diet

Fig. 2. Enlarged uterus of the piglet seen in Fig. 1

(0.5—1.3%) was unaltered zearalenone. The maximum concentration of zearanol found in the milk was 0.575—0.790 ppm. The toxins could already be detected in the milk samples 42—44 hr after ingestion of toxic diet, and at the latest, on the 5th day after the diet had been finished, *i.e.* the residual life of the toxin was at least 5 days. However, not all milk samples contained the metabolites.

Essential differences could not be found between toxin-extracting methods (precipitation with chloroform-acetone mixture; precipitation of centrifuged skimmed milk by acetone; partition of these fractions and of the butterfat by acetonitrile and n-hexane) used. Toxins could be detected in both butterfat and skimmed milk as well as in the precipitate produced by treatment with acetone. The reason of this fact may be due to the presence of the glucuronic acid adduct because treatment with beta-glucuronidase was not done.

Discussion

In our experiment, the zearalenone consumed with the feed was transmitted into the milk of lactating sows as zearalenol.

Zearalenol is produced by several fungi belonging to the group *Fusarium* (STIPANOVIC and SCHROEDER, 1975). It is formed by reduction of the

keto group of the 6th carbon atom of the lactone ring to hydroxyl group (C = O → C—OH). Alpha-zearalenol fed to ovarectomized female mice was three or four times as estrogenic and uterotropic as either zearalenone or beta-zearalenol. HAGLER et al. (1979) found that cultures of *F. roseum var. gibbosum* produced alpha-zearalenol besides zearalenone, but no beta-zearalenol. The uterotropic activity of beta-zearalenol produced artifically earlier by chemical methods had a slightly less estrogenic effect in mice than had zearalenone, but did not lose this property completely (PETERS, 1972; HALGER et al., 1979). Beta-zearalenol formed in biological way has been found in porcine urine (PATHRE et al., 1978; VÁNYI and SZIGETI, 1978), in milk of cow and ewe (HAGLER et al., 1980a), and in culture medium as a result of the zearalenone-transforming activity of *Candida tropicalis* (PALYUSIK et al., 1980).

According to the literature, the larger amount of the zearalenone consumed by sows is eliminated with the faeces (VÁNYI and SZIGETI, 1978). In accordance with this, our experiments suggest that only a small portion of zearalenone is secreted in the milk.

Toxin could not be found in several milk samples. The reason of this is still unknown for us.

Summary

The object of this study was to determine whether ingested zearalenone or its metabolites appears in the milk of sows.

Two lactating sows were fed a diet containing 40 ppm (40 mg/kg) crystalline zearalenone. The feeding started on the 8th day after parturition and lasted 9 days continuously. The piglets were excluded from the toxic diet.

The vulvas of female piglets nourished solely with milk became swollen and red on the 4th or 5th day of the experiment, then the swelling progressed gradually.

Laboratory analysis of the milk proved that zearalenone, though in an altered form, did appear in the milk. Beta-zearalenol was found in the largest quantity (corresponding to 82.0—86.1% of the toxin appeared in the sow milk), whereas alpha-zearalenol and zearalenone were found in smaller quantities, namely, 13.4—16.7% and or 0.5—1.3%, respectively. The highest concentration of zearalenol found in the milk was 0.575—0.790 ppm. The toxins could be detected in the milk samples as early as 42—44 hr after the start of the toxic diet, and its residue could still be detected as late as 5 days after the diet had been discontinued.

Zearalenone and beta-zearalenol are approximately equal in estrogenic activity, while alpha-zearalenol is three or four times as estrogenic as zearalenone.

Acknowledgements

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TERATOLOGICAL EXAMINATION OF THE EXPERIMENTAL FUNGICIDE DPX 3217 50 WP IN CFY RATS

By

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(Received October 30, 1978)

The experimental fungicide DPX 3217 50 WP (DPX; Du Pont) is chemically 2-cyano-N-(ethylaminocarbonyl)-2-(methoxyimino) acetamide. In Hungary, the approval of new agricultural chemicals depends on their standing a series of tests (Test System for Toxicological Evaluation of Pesticides, 1976), which include evaluation of teratogenicity in rats.

The present study is concerned with the influence of DPX on the rat embryo during the second quarter of gestation, *i.e.* during the early organogenic period, which in this species includes implantation of the embryo, formation of the three germinal layers, and of primordia (KREYBIG, 1968).

Materials and methods

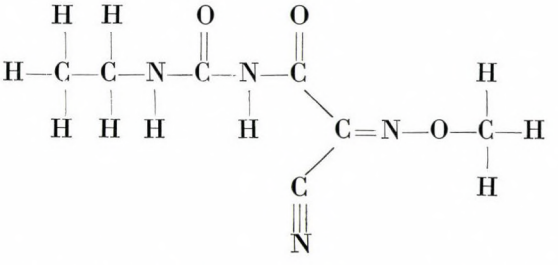
The test compound DPX contains 50% active substance (2-cyano-N-) ethylaminocarbonyl(-2-/methoxyimino/acetamide) and 50% other material.

It is a greyish-white, amorphous substance of non-characteristic odour; it can be easily powdered.

It is soluble in water up to 1000 mg/litre.

Empirical formula: $C_7H_{10}O_3N_4$.

Structural formula:



The pure active substance is an amorphous, pale pinkish, adhesive powder of non-characteristic odour.

Experimental animals

Adult CFY (LATI, Gödöllő, Hungary) albino rats of both sexes, weighing 185—230 g, were used. During the preparatory period (10—14 days) and subsequent experimental observation the animals were kept under controlled climatic conditions (22±2 °C, 65—70% relative humidity, with at least 8 changes of air per hr), and in artificial daylight for 12 hr daily. Plastic cages were used for accommodation, a commercial rat diet (KÁLLAI, 1968, 1975) was fed, and drinking water was supplied *ad libitum*.

Teratological examination

One male and a pair of females were placed in each cage. Three groups were formed, two for experimental treatment and one to serve as untreated control. The onset of pregnancy was determined from examination of vaginal smears every 8 hr, or presence of vaginal obstruction. After fertilization, the males were removed and the pregnant females were caged by fours. The experimental rats were given several oral doses of the test compound at 80.0 and 400.0 mg/kg level, respectively; the control were not treated. The applied doses corresponded to 5 and 25%, respectively, of the oral LD_{50} of DPX for the rat; the higher dose represented 80% of the no-effect level (BORDÁS et al., 1978).

At the 20th day of gestation all females were killed by ether overdose, and the fetal litters were examined. The following data were recorded for each litter: numbers of absorbed, live and dead fetuses, total weights of live and dead fetuses, mean weight of live fetuses, mean numbers of fetuses and implantations. Mothers and fetuses were examined for gross lesions.

From each female two fetuses were sampled at random for skeletal staining alizarin red R (SIMONS and VAN HORN, 1971).

Histological examination

Three fetuses from each group were fixed in 4% neutral formalin, embedded in paraffin, cut into 5—7 μm thick saggittal sections, and stained with haematoxylin and eosin (KISZELY and BARKA, 1958).

Mathematical-statistical evaluation

The significance of inter-group differences was assessed by variance analysis, as proposed by FINNEY (1952).

Results

The experimental data are presented in Tables I to IV. The control group included the litters of 13 mothers; these comprised 138 live fetuses, a single dead fetus, and 27 absorptions. The mean total weight of live fetuses and the

group average were 38.19 and 3.67 g, respectively. The mean fetal litter size was 10.6.

The group treated with 80 mg/kg DPX covered the litters of 12 mothers. The mean total weight of live fetuses was 47.88 g, the mean fetal weight 3.81 g, and the mean litter size was 12.58.

Table I

Absolute and mean fetal weights and distribution of live, absorbed and dead fetuses in the rat group used as untreated control

Dose mg/kg	Serial no. of litter	Absolute litter weights (g)		No. of fetuses per litter			Mean fetal weights per litter (g)[a]
		alive	dead	absorbed	alive	dead	
UNTREATED CONTROL	1	37.38	—	1	11	0	3.40
	2	30.52	—	0	7	0	4.36
	3	47.72	—	1	13	0	3.67
	4	43.12	—	1	12	0	3.60
	5	46.86	—	1	13	0	3.60
	6	58.60	—	1	14	0	4.19
	7	30.30	—	2	12	0	2.53
	8	47.54	—	1	13	0	3.66
	9[b]	12.00	—	2	8	0	1.50
	10	34.20	—	2	8	0	4.28
	11	10.34	0.42	9	3	1	3.45
	12	43.60	—	5	11	0	3.96
	13	54.30	—	1	13	0	4.18
Totals		496.48	0.42	27	138	1	—
Means		38.19	—	2.08	10.6	—	3.57
SD		±14.67	—	—	±3.2	—	±0.8

a, live fetuses; b, low litter weight

The litters of 17 mothers were studied at the 400 mg/kg dose level. In this group the live fetuses numbered 202, the absorbed ones 35, and a single dead fetus was found. The mean total and mean individual weights of live fetuses were 43.38 and 3.70 g, respectively. The mean litter size was 11.88.

Neither skeletal staining, nor histological examination revealed any abnormality in the fetuses of the treated rats (Figs 1—6).

Table II

Absolute and mean fetal weights and distribution of live, absorbed and dead fetuses in the rat group treated with 80 mg/kg DPX 3217 50 WP

Dose mg/kg	Serial no. of litter	Absolute litter weights (g)		No. of fetuses per litter			Mean fetal weights per litter (g)[a]
		alive	dead	absorbed	alive	dead	
80.0	1	51.30	—	0	14	0	3.67
	2	49.00	—	1	12	0	4.08
	3	56.00	—	1	14	0	4.00
	4	46.90	—	0	12	0	3.91
	5	47.62	—	3	14	0	3.40
	6	53.26	—	1	13	0	4.10
	7	33.20	—	0	8	0	4.15
	8	51.80	—	0	15	0	3.45
	9	52.42	—	1	14	0	3.74
	10	47.20	—	1	12	0	3.93
	11	58.12	—	0	15	0	3.87
	12	27.68	—	6	8	0	3.46
Totals		574.50	—	14	151	0	—
Means		47.88	—	1.17	12.58	—	3.81
	—	±8.91	—	—	±2.39	—	±0.3

a, live fetuses

Discussion

Tasting of the experimental fungicide DPX for teratogenicity in albino CFY rats has shown that neither 5%, nor 25% or the oral LD_{50} dose caused any fetal abnormality on repeated oral administration to the pregnant females during the second quarter of gestation. The stained fetal skeletons were in every respect normal, and the soft tissues showed no histopathological alteration.

No dose-response relationship could be established from the numbers of live, dead and absorbed vetuses in the two groups treated at different dose levels. The fetal weights and the total numbers of implantations were somewhat higher in the treated groups compared to the control ($P<0.05$).

The teratological evaluation of DPX had similarly advantageous results in the rat as in other species (VÁRNAGY and IMRE, 1978).

Table III

Absolute and mean fetal weights and distribution of live, absorbed and dead fetuses in the rat group treated with 400 mg/kg DPX 3217 50 WP

Dose mg/kg	Serial no. of litter	Absolute litter weights(g)		No. of fetuses per litter			Mean fetal weights per litter (g)[a]
		alive	dead	absorbed	live	dead	
	1	44.68	—	1	13	0	3.44
	2	40.68	—	1	13	0	3.13
	3	32.00	—	2	10	0	3.20
	4	45.10	—	5	12	0	3.76
	5	36.50	—	5	10	0	3.65
	6[b]	35.00	—	9	6	0	5.83
	7	52.82	—	0	14	0	3.77
400.00	8	53.10	—	0	13	0	4.08
	9	50.00	—	0	12	0	4.17
	10	28.54	—	0	8	0	3.57
	11	50.80	—	1	17	0	2.99
	12[d]	71.32	—	0	13	0	5.49
	13	37.76	2.10	4	12	0	3.14
	14	52.44	—	0	16	0	3.27
	15	47.80	—	2	13	0	3.67
	16	39.16	—	0	12	0	3.26
	17[e]	19.74	—	5	8	0	2.47
Totals		737.44	2.10	35	202	1	—
Means		43.38	—	2.06	11.88	—	3.70
SD		±11.86	—	—	±2.80	—	±0.8

a, live fetuses; b, fex live fetuses; d, high litter weight; e, low litter weight

Table IV

Teratological evaluation of the experimental fungicid DPX 3217 50 WP in CFY rats

Dose mg/kg	No. of pregnant females (litters)	Total weight of foetuses (group averages) (g)	Mean fetal weight (g)	Fetuses implant-absorbed			Mean litter sizes		Fetal lesions
				ed (mean ed	(mean)	mean abs.	live	dead	
Control	13	38.2 ± 14	3.57 ± 0.8	12.7	2.08	27	10.6	0.07	none
80	12	47.8 ± 9[a]	3.81 ± 0.3	13.7[a]	1.17	14	12.5	0.00	none
400	17	43.4 ± 12[2]	3.70 ± 0.8	13.9[a]	2.06	35	11.8	0.06	none

a, $P > 0.05$

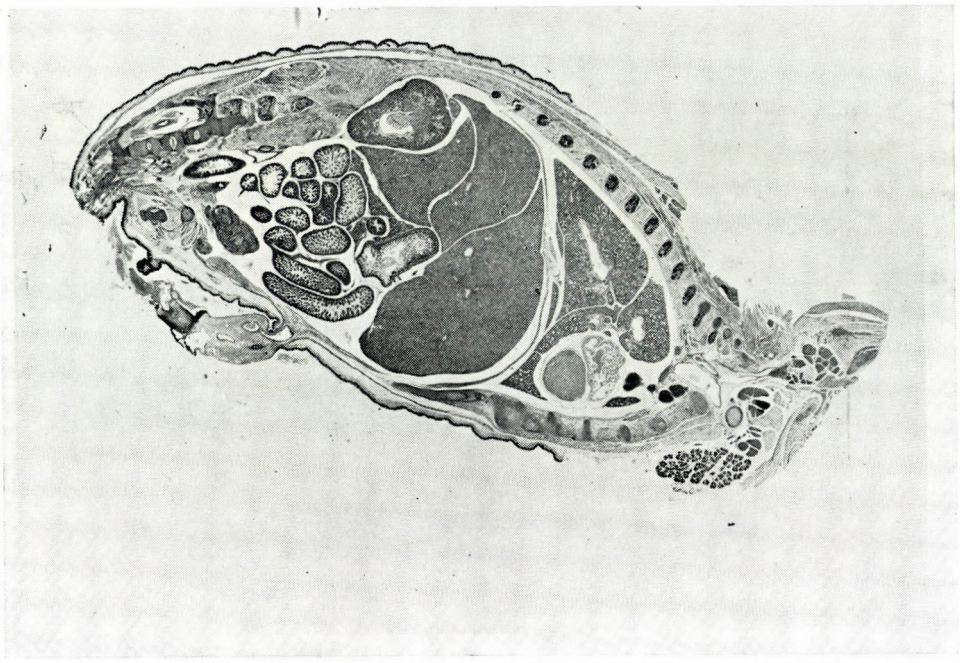


Fig. 1. Saggittal paraffin section from an untreated control fetus. H. & E., ×5.5

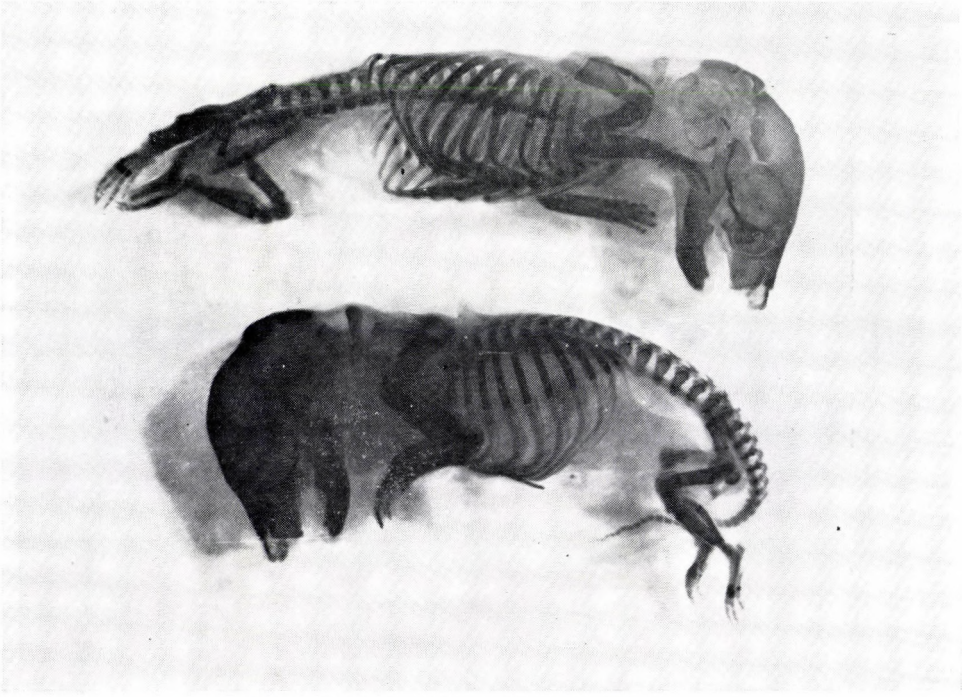


Fig. 2. Stained skeleton of a control fetus

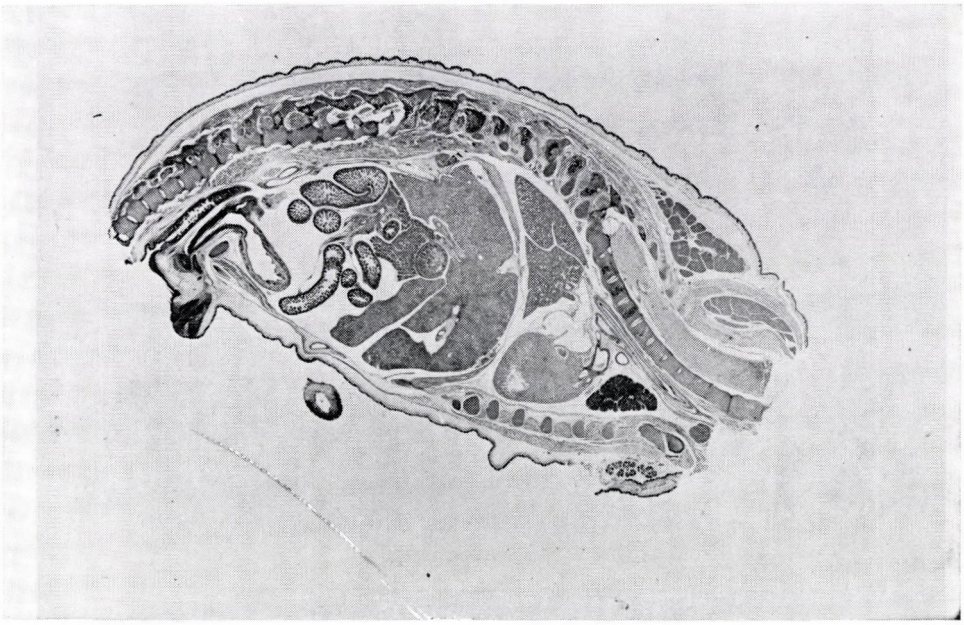


Fig. 3. Saggital paraffin section from a foetus in the 80 mg/kg dose group. H. & E., ×5.5

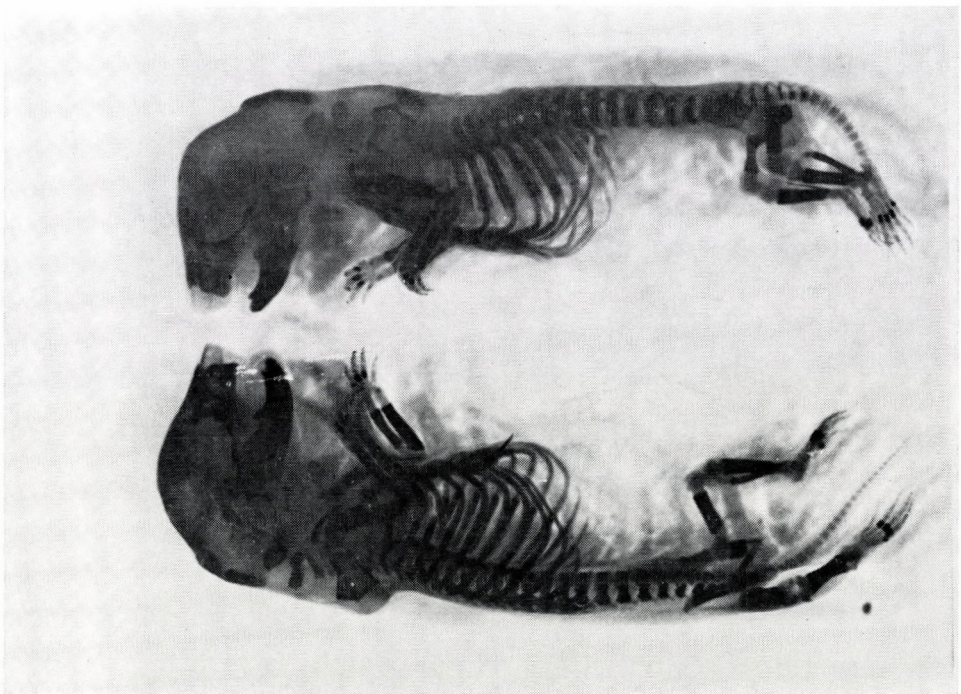


Fig. 4. Stained skeleton of a fetus the 80 mg/kg dose group

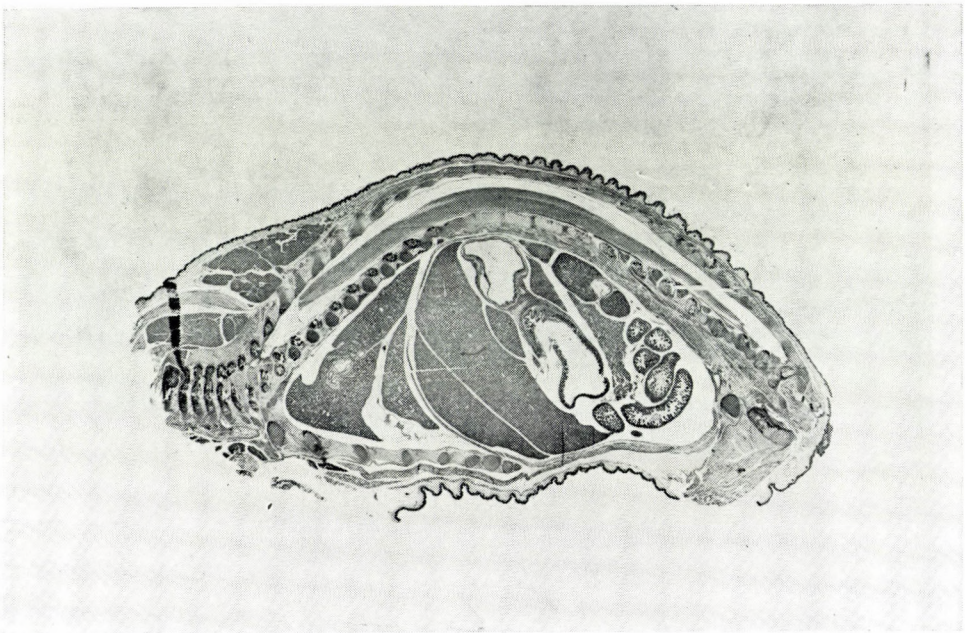


Fig. 5. Saggittal paraffin section from a fetus in the 400 mg/kg dose group. H. & E., ×5.5

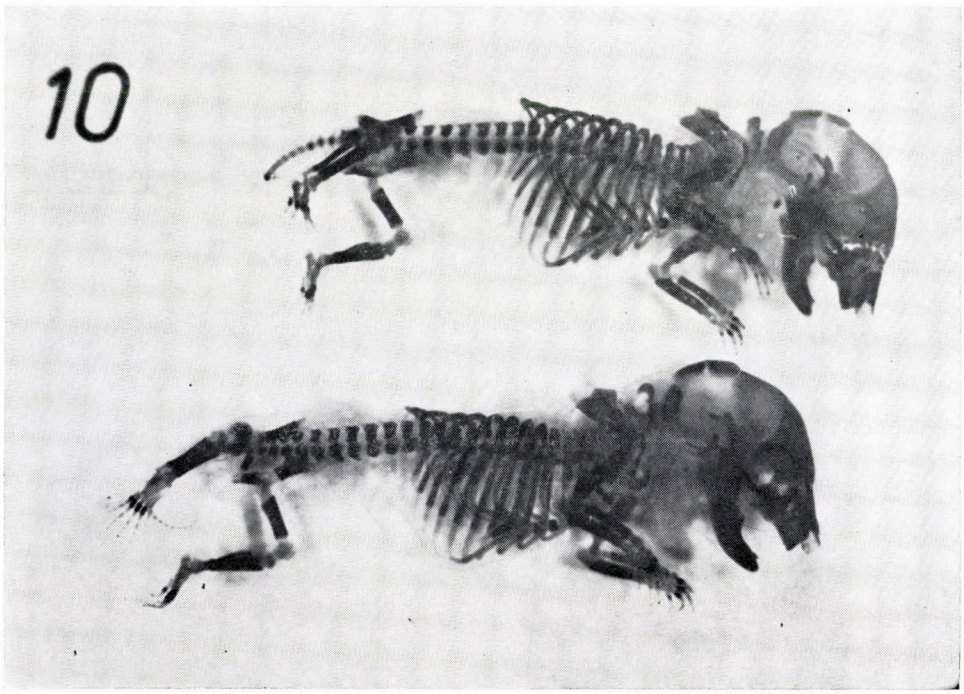


Fig. 6. Stained skeleton of a fetus from the 400 mg/kg dose group

Summary

The experimental fungicide DPX 3217 50 WP was tested for teratogenicity in albino CFY rats. Pregnant females were treated until days 6—10 of gestation with 80 and 400 mg/kg daily oral doses of the preparation, which corresponded to 5 and 25%, respectively, of its oral LD_{50} for the rat. All mothers were killed on the 20th day of pregnancy, and the fetal litter of each was examined for numbers of live, dead and absorbed fetuses, and total weights of live and dead fetuses; mean litter weight and litter size were also determined. The mother and the fetuses were examined for gross lesions. Alizarin red R was used for staining of fetal skeleton and haematoxylin-eosin for histopathological staing in general.

The applied doses caused no pathological alteration either in the skeleton or in the soft tissues of the fetuses.

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USE OF RECENT FETAL BONE STAINING TECHNIQUES IN THE EVALUATION OF PESTICIDE TERATOGENICITY

By

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(Received February 21, 1978)

The range and uses of chemicals have been tremendously expanded all over the worls during the last two decades. In agriculture, chemization has resulted in increased application of various fertilizers, pesticides, plastics and feed additives. Of these, pesticides are the subject of this report, with special regard to their evaluation for teratogenicity in laboratory animals, by skeletal staining techniques.

In addition to assays for acute, subacute and chronic toxicity, the toxicological study of pesticides includes investigations into the mechanism(s) of toxic action as well as examinations for possible sensitizing, mutagenic, teratogenic or blastomogenic effects, cumulative toxicity, reproductive toxicity, metabolism *etc.*

In Hungary, efforts have long been made to prevent the issue of teratogenic pesticides. Evaluation of teratogenicity is therefore particularly important. Under a recently-developed testing system (System of Toxicological Evaluation of Pesticides, 1977) the approval of any pesticide formula is subject to objective exclusion of its teratogenicity.

Many environmental chemicals possess teratogenic properties. Among others, aflatoxin B_1 has been shown to develop teratogenic action in the chicken (PLATT et al., 1962), rat (BUTLER and WIGGLESWORTH, 1966) and hamster (ELIS and DIPAULO, 1967); zinc in the rat (HURTLEY and SWENERTON, 1966) and hamster (FERM and CARPENTER, 1968); cadmium in the hamster (FERM and CARPENTER, 1967a); calcium in the chicken (GRABOWSKI, 1966); selenium in man (ROBERTSON, 1970), rat (ROSENFELD and BEATH, 1954), pig (WAHLSTROM and OLSON, 1959) and sheep (ROSENFELD and BEATH, 1947); lead in man (ANGLE and MCINTIRE, 1964) and hamster (FERM and CARPENTER, 1967b); organic mercury in rat (NONAKA, 1969) and man (KAKITA, 1961); nitrosamines in the rat, hamster and chicken (DIPAULO, 1969).

Various pesticides, too, have been shown to be teratogenic; 2, 4, 5-T (more precisely its 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin component) for the mouse (Commission on Pesticides, 1969), rat (COURTNEY et al., 1970), quail and chicken (LUTZ-OSTTERTAG and DIDIER, 1971); Simazin for the quail (DIDIER and LUTZ-OSTERTAG, 1972); 2, 4-D for the rat (Commission on Pesticides, 1969), quail and chicken (LUTZ-OSTERTAG and LUTZ, 1971); paraquat for the chicken and quail (LUTZ-OSTERTAG and HENOU, 1974); DDT for the chicken and quail (DAVID and LUTZ-OSTERTAG, 1972); endosulphan for the chicken and quail (LUTZ-OSTERTAG and KANTELIP, 1971); parathion for the chicken and quail (LUTZ-OSTERTAG et al., 1969); adrin for the chicken and quail (LUTZ-OSTERTAG and LUTZ, 1969); azinphos for the pheasant (LUTZ and LUTZ-OSTERTAG, 1971).

Staining procedures for fetal bone

Authors(s)	Fixative, and time (days) of fixation	Maceration in KOH % and time (days)	Staining: Alizarin red; concentration, time	Clearing: fluids and time (days)	Total time (days)
SCHULTZE, 1897	95% e, (+)	3× (+)	(+), (+)	(+), (+)	?
MALL, 1906	f. (30)	10 (+)	(+), (+)	95% e—g (4—60)	35—95
DAWSON, 1926	95% e (2—3)	1 (2—3)	1 : 10 000 in 1% KOH until stained	95% e—g (12—19)	16—25
LIPMAN, 1935	95% e (+)	2 (until clear)	(+), 1 day	95% e—g (8)	?
RICHMOND and BENEDETT, 1938	95% e (14), then 1% K_2CO_3 (28)	1 (10), then 20% g and 1% KOH	.01, 7—14 days	95% e—g H_2O (7)	67—74
CUMLEY et al., 1939	95% e (2—3)	1 (7)	(8), 3—12 hr	1% KOH (7); 95% e (4); toluene (4); naphthalene (4); anise oil (4)	33
WILLIAMS⊕, 1941	10% f (7)	2 (5—7)	drops in 2% KOH (24 hrs)	e (0.75) to methylsalicylate (3)	17—19
NOBACK and NOBACK, 1944	95% e (14—60)	2—4 with aliz. drops (3—21)	—	increasing conc. of g.	17—81
HOOD and NEIL 1948	95% e (3) or f (2)	2 (3), 5 (+)	(+), 6—12 hr	2% KOH (1) to g. (4—5)	12—13
SEDRA, 1950	10% f (7)	2% NaOH' (3—7)	(+), 12 hr	NaOH—g (6)	17—20
ST. AMAND and ST. AMAND, 1950	95% e or f (2)	boiling, 10 min	(+), (+)	(+), (+)	?
GREEN, 1952	No fixation	1 (5)	1% KOH' 0.5% aliz., 5 days	g (storage)	10
CRARY, 1962	No fixation	1% KOH' drops 5% aliz. red (3)' repeat (3)	—	alc; e; g (1 : 2 : 2) 1 day	7

STAPLES and SCHNELL, 1965	(+), then a (0.5)	1 (1.5) with 6 mg/l aliz.	—	Benzyl alc.; e; g (1 : 2 : 2), 12 hr		2.5
BURDI⊕, 1965	f	1 (3) with 2—3 a—e, 40 min.	—	e.g (1 :) until clear		8—12
JENSH and BRENT, 1966	10% f (7—14)	1 (3)	6 mg/1 (3)	Benzyl alc.; e; g; (1 : 2 : 2), 12 hr		11—18
SIMONS and VAN HORN, 1971	96% e (2)	0.5—1.0 (4—6)	drops in 1% KOH (5 days)	g		13—20
TIPTON and BURT, 1977	a (15 hr)	1.0 (1)	0.0025%, (1.5 days)	70%e g benzyl alc.	400 ml 400 ml 200 ml	11—18

(+), data not given; ⊕, toluidine blue staining omitted from the procedure. Abbreviations: a, acetone; e, ethanol; f, formalin; g, glycerol; alc., alcohol; aliz., alizarin red

Generally mouse, hamster and rat strains, as well as domestic chickens, pheasants and Japanese quails have been used for laboratory assays of teratogenicity. Deformities occurring during gestation become most conspicuously expressed in alterations of the skeletal system; these can be visualized by various bone staining techniques, of which the most important ones are listed in Table I.

Adaption of a method generally employed for avian bone staining (SIMONS and van HORN, 1971) to skeletal staining in rat embryo is reported, and a simple and relatively rapid technique, suitable for skeletal staining even after lasting incubation of the test material in formalin, is described.

Materials and methods

Procedure 1. CFY rat fetuses (LATI, Gödöllő) of 11—20 days' gestation were used. Prior to processing the fetuses were eviscerated, and their eyes and skin were removed. The specimens were subsequently processed essentially as proposed by SIMONS and Van HORN (1971), with some minor modifications. For staining of the cartilagineous tissues, the specimens were incubated for 24 hr in 80 ml 96% ethanol +20 ml 99.5% acetic acid + 15 mg alcian blue 8 GS, followed by dehydration in 2—3 changes of absolute ethanol for 96 hr. The dehydrated specimens were cleared for 5 hr in 2—3 changes of 0.5—1.0% KOH, depending on the age of the fetus. For bone staining, alizarin red S was employed in saturated aqueous solution, of which 2—3 drops were added to 100 ml KOH; if the colour had faded, more stain was added to obtain the desired shade. The staining time was 96 hr.

As a result of processing the cartilagineous tissue took on a blue colour, the bone tissue a red colour. The processed specimens can be stored for an unlimited time in 87% glycerol.

Procedure 2. Rat fetuses of similar age from the same breed as used in procedure 1 were prepared by evisceration and removal of the eyes and skin, and were fixed in 4% neutral formalin for 48 hr. It is also possible to remove the embryos directly into the fixing solution, and to prepare them afterwards. The fixed specimens were washed in tap water for 3 hr and subsequently the cartilagineous tissue was stained — if required — with alcian blue solution for 24 hr as above.

Dehydration in 2—3 changes of absolute ethanol followed for 60 hr. The dehydrated specimes were incubated in 2—3 changes of 1% KOH for 5 hr. Staining with alizarin red R was performed as in procedure 1, except that the stainig time was reduced to 80 hr.

The cartilagineous and skeletal tissues took on blue and red stain, respectively, and the stained specimens could be preserved in 87% glycerol for an unlimited time.

Results

Both procedures proved to be suitable for staining the cartilagineous and osseous tissues of fetal rats blue and red, respectively. If only bone staining is aimed at, processing of the cartilages can be omitted; in this case the procedure can be shortened by 24 hr. The stained fetal skeletons are well visible with the naked eye or the stereomicroscope, and yield photograpic pictures of good quality.

Discussion

The midified version of SIMONS and VAN HORN's technique, described in this paper as Procedure 1, is suitable for skeletal staining in fetal rat for the purpose of detecting pesticide teratogenicity. Procedure 1 requires much less time (about 220 hr) than the original fetal rat bone staining technique (about 320 hr), and the result is as clear-cut as in avian bone staining.

Procedure 2 is also time-saving; like procedure 1; it takes only about 220 hr. Furthermore, it makes possible a clear-cut skeletal staining also after lasting storage of the fetuses in formalin. This is particularly advantageous when large batches await processing.

The success of processing depends on the age of the embryo. Evisceration and removal of the skin and eyes are easily done until 10—12 days of gestation, but care should be taken to use an appropriate concentration of KOH, for even 1% KOH may cause over maceration of the soft parts, and give thereby disintegrate the fetus. Removal of the skin and cervical soft tissues, glands, *etc.* is considerably less easy in older fetuses, but it is indispensable for an appropriate staining of the dorsal and cervical vertebrae.

Summary

Two skeletal staining techniques are proposed for the demonstration of pesticide teratogenicity in the fetal rat. Both procedures are reliable and time-saving as compared to the previously published ones.

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INDEX

Bacteriologia

CZEGLÉDI-JANKÓ, G. and DANKÓ, Gy.: A New Microbiological Assay System for Antibacterial Drug Residues in Animal Organs. Estimation of Nigericin Residues 121

Virologia

MOCSÁRI, E.: Vaccination Experiments Against Transmissible Gastroenteritis (TGE) of Swine. VII. Immunoglobulin Characteristics of Antibodies in Milk of Sows Vaccinated with the "CKp" Strain of TGE Virus 131

KAPP, P. and LAPIS, K.: Reaction of Dogs Treated with Immunosuppressive Agents to Infection with the Virus of Rubarth Disease 145

Mycologia

PALYUSIK, M., HAGLER, W. M., HORVÁTH, L. and MIROCHA, C. J.: Biotransformation of Zearalenone to Zearalenol by *Candida tropicalis* 159

Physiologia

JÉCSAI Jolán, SZELÉNYI-GALÁNTAI Marianna, JUHÁSZ, B., SZEGEDI, B. and TELEKI Mária: Experimental Studies on Early Weaned Lambs. III. Haematological Appraisal of Protein and Amino-Acid Nutrition 167

TELEKI, Mária, JUHÁSZ, B., SZELÉNYI-GALÁNTAI Marianna, JÉCSAI Jolán and SZEGEDI, B.: Experimental Studies on Early Weaned Lambs. IV. Energy and Gas Metabolism in Early Weaned Lambs 177

HARASZTI, J., SZENCI, O., BÁRDOS Anna and BAKSAI-HORVÁTH Éva: Studies of Dietary Influence on the Health State of Preparturient Cows in Two Dairy Herds 189

HARASZTI, J., SZENCI, O., BAKSAI-HORVÁTH Éva and SOLTI, L.: Peripartal Blood Profile Studies in High-Production Dairy Cows with Special Regard to Reproductive Reactivation 197

HAGLER, W. M., DANKÓ, Gy., HORVÁTH, L., PALYUSIK, M. and MIROCHA, C. J.: Transmission of Zearalenone and its Metabolite into Ruminant Milk 209

PALYUSIK, M., HARRACH, B., MIROCHA, C. J. and PATHRE, S. V.: Transmission of Zearalenone and Zearalenol into Porcine Milk 217

VÁRNAGY, L. and IMRE, Rózsa: Teratological Examination of the Experimental Fungicide DPX 3217 50 WP in CFY Rats 223

Morphologia

VÁRNAGY, L.: Use of Recent Fetal Bone Staining Techniques in the Evaluation of Pesticide Teratogenicity 233

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РЕЗЮМЕ

НОВЫЙ МИКРОБИОЛОГИЧЕСКИЙ СПОСОБ ОПРЕДЕЛЕНИЯ ОСТАТКОВ АНТИБАКТЕРИАЛЬНЫХ СРЕДСТВ В ОРГАНАХ ЖИВОТНЫХ. ОПРЕДЕЛЕНИЕ ОСТАТКОВ НИГЕРИЦИНА

Г. ЦЕГЛЕДИ-ЯНКО и Дь. ДАНКО

Разработан новый микробиологический способ определения остатков антибактериального действия веществ в органах животных, обработанных нигерицином.

Мешающее действие ко-экстрактов, обычно наблюдающееся при обычной агардиффузионной реакции, авторы устранили.

Агарную пластинку заменили пластинкой из плексистекла, в которую врыли в 10 рядах по малых углублений. В ряды углублений вносили серию двухкратных разбавлений изучаемых образцов и референтного вещества. Пластинку потом покрыли питательной средой из агара, зараженной спорами **Bacillus subtilis**. Во время инкубации микроорганизмы размножаются за исключением тех углублений, в которых было еще достаточное количество нигерицина для торможения роста микроба. Основанием оценки уровня остатка вещества является сравнение ряда разбавления изучаемых образцов с референтным рядом разбавления действующего начала. Таким образом можно выявить остатки вещества, налицевствующего в концентрации 0,001 мг/кг.

ЭКСПЕРИМЕНТЫ ПО ВАКЦИНАЦИИ ПРОТИВ ВИРУСНОГО ПОНОСА СВИНЕЙ. VII. ИЗУЧЕНИЕ ИММУНОГЛОБУЛИНОВЫХ СВОЙСТВ АНТИТЕЛ МОЛОКА СВИНОМАТОК, ВАКЦИНИЗИРОВАННЫХ ШТАММОМ СКр ВИРУСА ВИРУСНОГО ПОНОСА СВИНЕЙ

Е. МОЧАРИ

Автор следил за формированием специфических антител вирусного поноса свиней трансмиссивного гастроэнтерита в молоке и крови свиноматок, вакцинировавшихся вирусным штаммом через рот, внутримышечно и выживших после естественной инфекции. Потом определил иммуноглобулиновые классы лактогенных антител против вирусного поноса свиней и их вируснейтрализирующую (ВН) активность.

Титр антител в молозиве как через рот, так и естественно заразившихся свиноматок был больше на 1 $\log_2$, чем в сыворотке. К 3—7-у дню после опороса титер антител в молочной сыворотке резко понизился (на 2—3 $\log_2$ (по сравнению с их титром в крови и до конца лактации остался на том же уровне. В сыворотке и молозиве привитых внутримышечно свиноматок титры антител были выше, чем таковые перорально привитых и естественно заразившихся животных. Но с первого дня лактации показатели титров антител у внутримышечно привитых свиноматок в молочной сыворотке начали резко уменьшаться и к 7—14-у дню они совсем исчезли.

В молоке через рот прививавшихся свиноматок — равно как и естественным образом заразившихся — специфическая ВН-активность была связана главным образом с I_gA.

Причной резкого понижения к 2—7-у дню после опороса титра антител в молочной сыворотке естественно заразившихся или перорально вакцинировавшихся свиноматок (на 2—3 $\log_2$ в отношении к титру в крови) явилось понижение концентрации I_gG и связанное с ним понижение уровня ВН-активности. После этого персистирующая до конца лактации ВН-активность против вируса вирусного поноса свиней налицевствует в первую очередь или исключительно за счет персистирующей концентрации I_gA.

У внутримышечно вакцинировавшихся свиноматок в молозиве и молочной сыворотке — в силу стимула парэнтерального антигена — образуются только антитела типа I_gA. Таким образом титер специфических антител в молочной сыворотке с понижением концентрации I_gG параллельно падает. Специфическая вируснейтрализирующая активность в зависимости от начального титра между 7—14-ым днем лактации, когда в молочной сыворотке концентрация I_gG падает под определенный уровень, обычно исчезает.

РЕАКЦИЯ ИММУНОСУПРЕССИВНЫМ СРЕДСТВОМ ОБРАБОТАННЫХ СОБАК НА ЗАРАЖЕНИЕ ВИРУСОМ БОЛЕЗНИ РУБАРТА

П. КАПП и К. ЛАПИШ

Авторами раньше уже доказано, что при вирусном гепатите собак этиологический фактор размножается главным образом в синусоидах печени, в клетках ретикуло-гистиоцитарной системы (РГС) и на этом основании предполагали, что в тяжелом или легком течении болезни важную роль играет состояние клеток РГС. Ради доказательства этого предположения 4-х восприимчивых щенков того же помета заразили внутримышечно одинаковой дозой вируса болезни Рубарта. Двух щенков кроме этого обработали одновременно цитостатическим веществом (ликуримом), повреждающим целлюлярную клеточную систему. У зараженных животных при помощи биопсии большеберцовой кости следили за изменениями в печени и кровяной картине. У обработанных ликуримом животных количество белых кровяных клеток (главным образом лимфоцитов) резко уменьшилось, в купферовых клетках печени наступили повреждения. Среди 4-х зараженных щенков два — обработанные иммуносупрессивным веществом — 5 дней после заражения погибли от острого вирусного гепатита (острая дистрофия печени). Остальных два щенка после инфекции выжили.

Эксперименты на щенках того же помета показали, что летальный острый вирусный гепатит наступает, как правило, в том случае, если в момент нападения вируса клетки РГС находятся из-за любой причины в блокированном состоянии.

БИОТРАНСФОРМАЦИЯ ЗЕАРАЛЕНОНА В ЗЕАРАЛЕНОЛ ПРИ ПОМОЩИ ГРИБКА CANDIDA TROPICALIS

М. ПАЛЮШИК, В. М. ХАГЛЕР, Л. ХОРВАТ и Ц. Й. МИРОХА

В аэробной жидкой культуре при 32 °С C. tropicalis редуцирует 80% добавленного зеараленона в зеараленол (с 89%-ами бета-зеараленола и 11%-ами альфа-зеараленола — диастереомером, обнаруженным в культурах зеараленон продуцирующего грибка Fusarium roseum "Gibbosum"). Только 20% добавленного зеараленона остается неизмененным. Других метаболитов в культуре не обнаружено. Трансформация завершилась в течение двух суток, но превращение основного процента соединения завершилось уже в течение двоих суток.

Эстрогенность бета-зеараленола равняется примерно таковой зеараленона, тогда как она же альфа-зеараленола в четыре раза крепче зеараленона. Анализ осуществляется при помощи газ-жидкость хроматографией на 3%-ном QF-1 и жидкостной хроматографией высокой чувствительности на одной оборотно-фазовой колонке C_{18} и проявлен массовой спектрометрией. Результаты подсказывают, что ферментацией засоренного зеараленоном корма нельзя избавиться его токсичности. Дальше, возможность массовой трансформации зеараленона в зеараленол естественной микрофлорой или микрофауной требует разработки надежных методов выявления этого метаболита. Метаболизация ^{3}H-зеараленона в ^{3}H-зеараленол грибком C. tropicalis при комнатной температуре.

ИЗУЧЕНИЕ ДЕЙСТВИЯ КОРМЛЕНИЯ НА СОСТОЯНИЕ СТЕЛЬНЫХ КОРОВ НА ДВУХ МОЛОЧНЫХ ФЕРМАХ

Я. ХАРАСТИ, О. СЕНЦИ, АННА БАРДОШ и ЕВА БАКШАИ-ХОРВАТ

Материал для исследования при помощи биопсии брали от 27 коров 1, 2 и 3 недели до отела в поголовьях гольштеин-фриз и венгерской пестрой. На сухостое коровы обоих поголовьев снабжались достаточным количеством кукурузного силоса, концентратов и малым количеством сена. Кукукрузный силос поголовья гольштеия-фриз был плохо консервированным, содержал много масляной кислоты. Среди образцов от 15 животных в 8 образцах в печеночных клетках обнаружена жирная инфильтрация; это изменение в образцах от животных венгерской пестрой породы обнаружено в 5 случаях от 12-и. Животные были лучше средней упитанности, их не выпасали, а содержали в стойлах, без моциона. Изучение разных показателей крови двух клинически больных животных показало сдвиги значения трансаминаз, сахара, каротина, концентрации кальция и фосфора. В поголовье гольштеин-фриз среди отелившихся 272 коров задержание последа, заболевания матки и кистозность яичника, дальше, маститы наблюдались у 13,2, 33 и 10%-ов животных соответственно. Пять месяцев после отела 26% коров были еще холостыми.

ИЗУЧЕНИЕ ПРОФИЛЯ КРОВИ У МНОГОМОЛОЧНЫХ КОРОВ В ПЕРИОД ОТЕЛА С ОСОБЫМ ВНИМАНИЕМ НА ВОЗОБНОВЛЕНИЕ ПОЛОВОЙ АКТИВНОСТИ

Я. ХАРАСТИ, О. СЕНЦИ, ЕВА БАКШАИ-ХОРВАТ и Л. ШОЛТИ

Авторами изучались параметры крови 20 многомолочных коров породы гольштеин-фриз, старше 5 лет, в период 8 дней до и 10 дней после отела. Среди животных после отела 6 заболело (не встало), 14 же были бессимптомными. У заболевших коров сильно повысилась активность ГОТ, ГПТ, заметно повысилась она у ЛДХ, концентрация кальция была низкой, а таковая фосфора была очень низкой по сравнению с здоровыми животными. Уровень мочевого азота в крови в днях до отела у больных и здоровых животных был на 30—40 и 10—20% соответственно выше среднего. Бросался в глаза низкий уровень холестерина у всех животных и тот факт, что изучавшиеся параметры за исключением активности ГПТ и неорганического азота даже у бессимптомных животных показывали меньший-больший сдвиг.

В период от 21-ого по 35-й день после отела уровень прогестерона был в большинстве случаев минимальным, характерный для минимума овариектомизированных коров. Среди изучавшихся 20 коров в период от 60-ого по 140-й день после отела обеременились 6 коров (30%); в период между 190-м и 240-м днем 9 коров были еще холостыми (45%); после некоторого времени 5 животных (25%) вынуждено убито. Холостые животные даже после повторного лечения не обеременялись, перебои их половой деятельности отражались в увеличении так наз. «пустых дней» (open days).

ПЕРЕХОД ЗЕАРАЛЕНОНА И ЕГО МЕТАБОЛИТОВ В МОЛОКО У ЖВАЧНЫХ

В. М. ХАГЛЕР, ДЬ. ДАНКО, Л. ХОРВАТ, М. ПАЛЮШИК и Ц. Й. МИРОХА

Пять грам кристаллического зеараленона с кормом и 1,8 г зеараленона в 96%-ном этаноле введено корове и овцематке соответственно. В некоторых экстрактах из молока, анализированных методом оборотно-фазовой жидкостной хроматографии высокой чувствительности в некоторых случаях выявлены следы (примерно 0,0005 ppm) зеараленона и бета зеараленола. Зеараленон и бета зеараленол налицевоствовали и в некоторых экстрактах из молока овцематки в концентрации 0,001—0,002 ppm. Экстракты из мочи овцематки содержали эти соединения в концентрации 0,20—0,050 ppm, выявимые и тонко-слойной хроматографией, если зеараленон скормили не раньше 24-х часов. Ярочка овцематки кормилась молоком, неиспользованным для экстракции и показывала выраженные признаки гиперэстрогенизма — резкое увеличение вульвы — примерно в течение 10 дней от обработки. Аналитический метод обратно-фазовой жидкостной хроматографии высокой чувствительности является очень чувствительным и специфическим способом анализа этих метаболитов в молоке, поскольку граница их выявления находится ниже 0,0005 ppm.

ЭКСПЕРИМЕНТЫ ПО РАННЕЙ ОТБИВКЕ ИСКУССТВЕННО ВЫРАЩЕННЫХ ЯГНЯТ

III. ОПРЕДЕЛЕНИЕ ПОТРЕБНОСТИ ИСКУССТВЕННО ВЫРАЩЕННЫХ ЯГНЯТ В БЕЛКАХ И АМИНОКИСЛОТАХ НА ОСНОВАНИИ ПАРАМЕТРОВ КРОВИ

ЙОЛАН ЕЧЕИ, МАРИАННА СЕЛЕНИ-ГАЛАНТАИ, Б. ЮХАС, Б. СЕГЕДИ и МАРИЯ ТЕЛЕКИ

На трех группах животных изучалась потребность искусственно выращенных ягнят в белках и аминокислотах. Группы отличались друг от друга в дозировке заменителя молока и времени отбивки.

Степень потребности в аминокислотах определяли на основании концентрации свободного метионина, лизина и определением азота всех аминокислот в образцах крови, полученных в одинаковые сроки после кормления животных.

В первой группе ягнят постепенное изъятие из рациона заменителя молока с успехом координировалось с повышающимся приемом плотного корма — источника протеина и аминокислот — результатом чего и явилось бесперебойное всасывание и усвоение этих эсенциальных питательных веществ в период изменения рациона. Параметры крови до 23-дневного возраста изменялись согласно количеству потребленных белков и аминокислот. В группе II наблюдалась такая же тенденция как и в группе I, но она менялась количественно в согласии с технологией кормления. В группе III после 7 дней в силу подкормки аминокислотами (заменитель молока) повысилась концентрация лизина и метионина в крови. Но из-за дефицитности в снабжении кормом значение подкормки не реализовалось надлежащим образом. Улучшение состояния наступает только позже, после развития функции рубца и поедания требуемого количества плотного корма.

Между количеством принятого лизина и метионина и их концентрацией в крови обнаружена умеренно тесная взаимосвязь ($r = 0{,}65 - 0{,}57$).

Ради оценки снабженности белками, следили за изменениями содержания в крови всех белков и остаточного азота. Между количеством принятых с кормом белков и концентрацией остаточного азота в крови наблюдалась умеренно тесная взаимосвязь ($r = 0{,}61$).

Согласно данным исследований при ранней отбивке ягнят необходимо обеспечить надлежащее снабжение белками и аминокислотами. В данных экспериментах при ранней отбивке требуемое снабжение белками и аминокислотами удалось обеспечить таким образом, если молоко изъяли из рациона постепенно (в возрасте 12—32 дней). При таком режиме ягненок получает достаточное количество качественных белков и аминокислот, особенно в критический период смены кормов.

ЭКСПЕРИМЕНТЫ ПО РАННЕЙ ОТБИВКЕ ИСКУССТВЕННО ВЫРАЩЕННЫХ ЯГНЯТ

IV. ОБМЕН ЭНЕРГИИ И ГАЗООБМЕН У СКОРО ОТБИТЫХ ЯГНЯТ

МАРИЯ ТЕЛЕКИ, Б. ЮХАС, МАРИЯННА СЕЛЕНИ-ГАЛАНТАИ, ЙОЛАН ЕЧАИ и Б. СЕГЕДИ

У ягнят трех групп, отбитых в дневном возрасте и выращенных на искусственном корме, изучали обмен энергии и газообмен.

Применявшийся заменитель молока скармливался в 20%-ном разбавлении и его литр содержал 1450 Kcal валовой энергии, 95% которой переваримо. Килограмм плотных кормов содержал 3800—4100 Kcal. 92,5 и 78,8% энергии заменителя молока и плотных кормов соответственно было усвоимо.

Потребность в поддерживающей энергии ягнят колебалась в пределах 109—76 Kcal/день/кг и с возрастом она уменьшилась. Согласно этому понизилась отдача тепла/час. Этот показатель равнялся 4,51—3,23 Kcal.

Энергия, накопленная в привесе от 720 Kcal понизилась до 0 Kcal, больше того, в 3-м и 5-м этапах экспериментов получен отрицательный баланс энергии, когда ягнята потребляли меньше энергии, чем потребность поддерживающей таковой.

С возрастом количество энергии, вмещающейся в белковом содержимом привеса — взамен энергии в жире — повысилось. Объяснением этого, могласно авторам, является снижение интенсивности кормления. Образование протеина и тем самым накопление энергии, вмещающейся в нем, даже при отрицательном балансе энергии не прекратилось. Для образования 1 г-а белка ягнята потребляли 10,4—24,0 Kcal полезной энергии.

ПЕРЕХОД ЗЕАРАЛЕНОНА И ЗЕАРАЛЕНОЛА В МОЛОКО СВИНОМАТКИ

М. ПАЛЮШИК, Б. ХАРРАХ, Ц. И. МИРОХА и С. В. ПАТРЕ

Задачей исследований явилось определение того, что введенный через рот зеараленон или его метаболиты переходят-ли в молоко свиноматки.

Две лактирующих свиноматки кормились кормом, рацион которого содержал 40 ppm (40 мг/кг) кристаллического зеараленона. Кормление начали на 8-й день после опороса и оно продолжалось 9 дней без перерыва. Поросята не допускались к токсическому корму.

Вульва поросят женского пола 4—5-ого дня эксперимента стала постепенно набухать.

Лабораторный анализ молока показал, что зеараленон — хотя в измененной форме — но появляется в молоке. Больше всего было в молоке бета-зеараленола (примерно 82,0—86,1% от всего принятого токсина), тогда как альфа-зеараленол и зеараленон обнаруживались в малом соотношении 13,4—16,7 и 0,5—1,3% соответственно. Наибольшая концентрация зеараленола в молоке равнялась 0,575—0,790 ppm. Токсины обнаруживались в молоке уже к 42—44-у часу после начала скармливания токсического корма и его остатки налицевствевали в молоке 5 дней после закончения кормления свиноматок ими.

Эстрогенное действие зеараленона и бета-зеараленола одинаково сильно, тогда как оно альфа-зеараленона три или четыре раза сильнее зеараленона.

ТЕРАТОЛОГИЧЕСКОЕ ИЗУЧЕНИЕ ФУНГИЦИДНОГО СОЕДИНЕНИЯ DPX 3217 50 WP [2-ЦИАНО-N-(ЭТИЛАМИНОКАРБОНИЛ)-2-МЕТОКСИЛАМИНО-АЦЕТАЛИД] НА КРЫСАХ

Л. ВАРНАДЬ и И. РОЖА

Докладывается об экспериментальном изучении тератологического действия фунгицидного средства **DPX** 3217 50 **WP** на крысах албино **CFY**. С 6-ого по 10-й деиь беременности крысихи получали при помощи желудочного зонда ежедневно по 0,00, 80,0 и 400,0 мг/кг препарата соответственно. Избранные дозы соответствовали 1/20-й и 1/4-и показателя LD_{50} через рот. Оценка действия осуществлялась на 20-й день беременности.

В исследованиях измерили суммарный вес живых и мертвых плодов у каждой крысихи, определяли количество резорбировавшихся, живых и мертвых плодов, вычислили средний вес плодов и их среднее количество на одну крысиху.

В исследованиях крысихи и их плоды подвергались патологоанатомическому изучению. Для выявления эвентуальных изменений в костях скелета крысят применяли окрашивание ализариновой красной и готовили гистологические срезы, окрашенные гематоксилин-эозином.

Применявшиеся дозы соединения в данных исследования не вызывали тератологического действия, проявляющегося в изменениях костей или паренхиматозных органов развивающихся крысят.

НОВЫЕ ВОЗМОЖНОСТИ В ОКРАШИВАНИИ ЭМБРИОНАЛЬНОГО СКЕЛЕТА В ИЗУЧЕНИИ ТЕРАТОЛОГИЧЕСКОГО ДЕЙСТВИЯ ПЕСТИЦИДОВ

Л. ВАРНАДЬ

Дается краткий обзор токсикологических исследований с пестицидами на животных. В связи с этим указывается на значение тератологических исследований при определении возможного вредного действия химических средств в том числе пестицидов. В работе описываются два метода окрашивания скелета крысиных плодов, удобных для быстрого и надежного выявления тератогенных изменений. Дается перечень досели описанных важнейших методов окрашивания эмбрионального скелета, на фоне чего становятся очевидными преимущества предлагаемых автором методов окрашивания.

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IDENTIFICATION OF GENERA IN THE FAMILY OF *ENTEROBACTERIACEAE* BY A U-TUBE MULTITEST METHOD IN FOOD MICROBIOLOGY

By

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(Received March 12, 1979)

Polytropic media are used for differentiation of genera in the family of *Enterobacteriaceae*. These media include some sugar ingredients for receiving information about the characteristic decomposition of biochemical reactions.

A method to be used in food microbiology should present results within 24 h. It should be simple and convenient to perform, the inherent errors should be minimum, the method should be reproducible and the whole procedure should be cheap (WHO, 1976).

The general principle of the polytropic media is mostly based on the decomposition of glucose, lactose, sucrose, urea and the production of H_2S. CHRISTENSEN (1946), SULKIN and WILLETT (1940), HAJNA (1945), KNOX (1949), KOHN (1954), TAYLOR and SILLIKER (1958), RODLER et al. (1960), LÁNYI and ÁDÁM (1960), GALTON et al. (1968), KAUFFMANN (1966), GRUNBERG et al. (1969), NÓGRÁDY and RÓDLER (1954), EDWARDS and EWING (1972), TOMFOHRDE et al. (1973), dealt with different types of multitest methods. TAKÁCS (1964) modified the original medium of RUSSEL (1911), KLIGLER (1917) and KOHN (1917). WIESNER (1970) proposed a method for identification of coliforms in milk, using a special polytropic medium.

Recently the "Enterotube" Roche is available on the market as a sterile multimedia tube for the rapid differential identification of Gram-negative bacteria *(Enterobacteriaceae)*. The tube consists of eight different conventional media and an enclosed inoculating needle. The multimedia tube has a number of advantages, however, its availability is limited.

IMViC tests are commonly used to identify lactose fermenters. However, these tests are insufficient to make difference among all the genera in the *Enterobacteriaceae* family. We prepared therefore a modified Wiesner's polytropic medium in which H_2S, motility and lysine-decarboxylase tests are included.

Materials and methods

Samples

Raw milk samples were collected from farms. The raw meat samples were taken from cattle, pigs and poultry slaughterhouses by surface-sampling method. Surfaces of the carcasses were tested with sterile swabs which sub-

sequently were kept in buffered peptone water for pre-enrichment and resuscitation for 18—24 h at 30 °C.

Composition of the polytrop medium. Different media were prepared and layered in the U tube. The basic medium was the semisolid SIM-agar (SIM = sulphide, indole, motility). In one wing of the U tube lysine-decarboxylase medium and in the other wing methyl-red (MR) and Voges-Proskauer (VP) medium were poured. The composition of the SIM medium was: Witte peptone 18.0 g, beef extract 8.0 g, ferrous ammoniumsulphate 0.2 g; sodium thiosulhpate 0.3 g; sodium chloride 3.0 g; agar 7.0 g; distilled water 1000 ml. First gelatin was soaked in the water, the other ingredients were added thereafter, and the mixture was heated until the ingredients completely dissolved. pH was adjusted to 7.2 and the medium was autoclaved at 121 °C for 10 min.

The ingredients of lysine-decarboxylase medium were: bactopeptone 4.5 g, beef extract 3.5 g, glucose 1.5 g; pyridoxinum chloride (vitamin B_6) 0.006 g; 0.2% bromcresol purple solution 10.0 ml; L-lysine-monochloride 10.0 g; distilled water 1000 ml. pH was adjusted to 5.5 ± 0.1. Finally, 2 ml volumes of the medium were distributed in small tubes and sterilized in the autoclave at 115 °C for 10 min. The VP medium was prepared according to the Barritt method.

Inoculation technique

VRB agar (violet red bile agar) and Drigalski agar plates were inoculated with 0.1 ml of inoculum and spread over the surface of agar and incubated at 30 °C for 24 h. Then single colonies were picked up from the plates (VRB or Drigalski) and the polytropic media in the U tube were stabbed on both wings of the tube with a sterile straight wire. The stab culture was made until the SIM medium. Incubation was at 30 °C and the results checked daily up to 5 days.

Results

After an incubation of 12—24 h at 30 °C it became possible to observe the production of H_2S, indole and the motility of the organisms in the SIM medium. In the positive cases the decarboxylation of lysine can be observed by its purple colour and pH rose to 6.5 or above. The MR test was checked directly, however, VP-positive strains were to be identified by adding of 1.0 ml alcoholic alpha-naphtol and 0.4 ml potassium hydroxide from a 40% solution. Positive reaction was indicated by a scarlet colour change of the medium (Fig. 1).

The U-tube multitest method provides the possibility for the identification of those genera in the family of *Enterobacteriaceae* which were difficult to differentiate from each other by the IMViC tests alone (Table I).

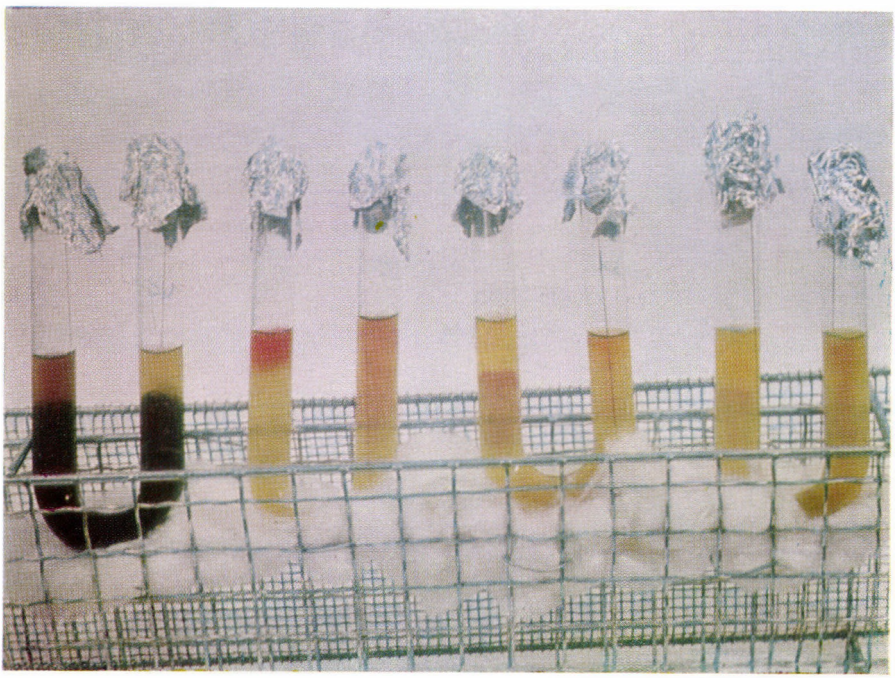

Fig. 1. Biochemical reactions of *Salmonella*, *Escherichia*, *Klebsiella* and *Enterobacter* in the polytropic medium filled in U-tubes

Table I

Differentiation of groups of coliforms including *Salmonella* and *Arizona* according to the IMViC-reactions

Genera	I	M	V	C
Escherichia	+	+	—	—
Citrobacter	—	+	—	+
Salmonella (*Arizona* *Salmonella* Subgenus III)	—	+	—	+
Enterobacter	—	—	+	+
Klebsiella	—	—	+	+
Hafnia (30 °C)	—	—	(+)	+

Table I shows that the behaviour of *Citrobacter* and *Salmonella* as well as that of *Arizona* and *Klebsiella, Enterobacter* and *Hafnia* genera is the same *i.e.* we cannot make difference among them by the IMViC tests.

Table II

Differentiation of coliforms from *Salmonella* and *Arizona* according the SIMoVLyM-reactions

Genera	S	I	Mo	V	Ly	M
Escherichia	—	+	+	—	(+)	+
Citrobacter	(+)	—	+	—	—	+
Salmonella (Arizona)	+	—	+	—	+	+
Enterobacter aerogenes	—	—	+	+	+	—
En. cloacae	—	—	+	+	—	—
En. liquefaciens						
Klebsiella	—	—	—	+	+	—
Hafnia (30 °C)	(—)	—	+	(+)	+	—

Explanations: Reaction detected: I, indole; M, methyl red; V, Voges—Proskauer; C, citrate; S, H_2S; Mo, motility; Ly, lysine decarboxylase. + positive; (+) mostly positive; — negative; (—) mostly negative.

In Table II is seen that by the U-tube multitest method it is easy to differentiate *Citrobacter* from *Salmonella* and *Klebsiella* from *Enterobacter*.

Discussion

The U-tube multitest method was found as one of the best methods for identifying and differentiating most of the genera of the family of *Enterobacteriaceae*. The method is not time- and work-consuming, and it is cheap because the most important biochemical reactions need a single tube. It is useful for salmonella examinations in food microbiology as well.

Summary

A modified U-tube method working with a new polytropic medium has been developed for the routine differentiation of genera within the *Enterobacteriaceae* family. The medium containing Voges–Proskauer as a component, indicates H_2S and indole decomposition, motility and lysine decarboxylase activity. The method is superior to the IMViC reactions by differentiating *Escherichia*, *Citrobacter*, *Salmonella-Arizona*, *Enterobacter*, *Hafnia*, and *Klebsiella* from one another. The differentiation is quick, cheap, and easy to use. The results are reproducible.

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ISOLATION OF AN ADENOVIRUS FROM RABBITS WITH DIARRHOEA

By

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(Received March 16, 1979)

Bacteriological examinations and virus isolation experiments were performed on organ samples derived from a rabbit flock with a history of recurrent diarrhoea among weanlings. This condition has been among the main causes of losses in rabbit flocks.

The pathogenesis of weanling rabbit diarrhoea has been extensively studied during the recent years, and authors generally agree that the disease is due to excess multiplication of *Escherichia coli* bacteria in the small and large intestine. The symptoms last 1—2 days and may be lethal in 15—25% of 5—8 weeks old weanling rabbits (Weber and Hoffmann, 1973; Hoffmann et al., 1973; Rossi and Mandelli, 1974; Vetési and Kutas, 1974; Sinkovics, 1976).

The main symptom is diarrhoea. Postmortem examination characteristically reveals thin caecal contents and atony of the small intestines. Gross inflammatory lesions (hyperaemia) and/or haemorrhages are seldom seen. Bacteriologically, an excess multiplication of *E. coli* is detectable exclusively in the small and large intestines. The parenchymatous organs are bacteriologically negative. Although the *E. coli* counts are always pathologically exalted (10^8—10^9/g) in both the small and the large intestine, the disease cannot be reproduced by oral transfer of the *E. coli* isolates to susceptible rabbits (Prohászka, 1972). This has suggested the involvement of certain unknown pathogenetic factors in the condition.

Materials and methods

The examinations reported in this paper were carried out in a large rabbit production unit built in the pavilion system. Broiler rabbit production was based on a production flock of 11,000 females on a continuous schedule. The average flock size was 110,000 rabbits, and the annual broiler rabbit output was up to 400,000. The animals were fed exclusively on a granulated rabbit diet prepared in the farm's own feed mill. The feed was supplied *ad libitum* in all age groups. The young rabbits were weaned at 4—5 weeks of age, then were grouped and transferred into cages with wire bottom. Diarrhoea set in shortly after establishment in the broiler rearing premises, and losses reached peak (10—15% mortality) at 6—8 weeks of age. Older animals were never involved by the disease.

The weaned rabbits were divided into three groups for experimental study, *i.e.*

A) apparently healthy weanlings, with no clinical sign of disease,

B) weanlings in the initial stage of disease, with a slight diarrhoea,

C) severely ill or premortal animals, or carcases.

The live rabbits were exsanguinated by cardiac puncture, the carcases were autopsied. The dry matter in caecal contents was determined. The bacteriological examination of the intestinal contents included only determination of the *E. coli* counts by titration procedure, for previous studies had shown that the changes of these are in themselves conclusive of the pathogenesis (PROHÁSZKA, 1972).

Mucosal smears taken from the jejunum and ileum were used for detection of coccidia by direct microscopic examination.

Virus isolation was attempted from various organs of animals killed in the initial stage of disease, *i.e.* while isolation of bacteria from organs still failed. As test material 20% suspensions of spleen, kidney, lung intestinal wall and intestinal content were used. The organ homogenates were suspended in Hanks' solution containing 100 μg/ml streptomycin, 50 μg/ml kanamycin and 50 μg/ml mycostatin.

Tissue cultures. Primary rabbit kidney epithelial cell cultures were used for virus isolation. Confluent monolayers were inoculated and were examined for CPE on seven consecutive days.

Acridine orange staining technique was performed as described in a previous paper (BODON, 1967).

Virus titration. The nutrient media of cell cultures exhibiting intranuclear inclusions in stained preparations were used for further passages in primary rabbit kidney cell cultures.

Tenfold serial dilutions of the virus material were prepared in Hanks' solution, and 0.2 ml inocula of each dilution were transferred to two Leighton tubes. Six or seven days after infection the coverslip cultures were stained with acridine orange. The highest dilution still giving rise to Cowdry type-A intranuclear inclusions was regarded as the titre of the virus.

Host tissue specificity tests. Apart from the primary rabbit kidney cell culture, primary pig kidney, calf kidney, calf testicle cell cultures, the human embryonic lung (HEL) cell line, and the pig kidney cell line PK-15 were inoculated with virus material.

Resistance and stability tests. Resistance to chloroform, trypsin, Tween 80, pH 3.0 and heat (1 h at 37 and 50 °C) were tested by the usual methods (FELDMAN and WANG, 1961; DINTER, 1963; CARTWRIGHT and THORNE, 1959; HAMPARIAN et al., 1963).

The viral nucleic acid was typed by two methods, *viz.*

1. by establishment of the UV spectrum of intranuclear inclusions stained with acridine orange, and

2. by the iododeoxyuridine (IDU) test. The maintenance medium was decanted from the monolayer cultures and replaced with 1.5 ml IDU, at 50 or

100 μg/ml concentration. The cultures were incubated for 1—2 h at 37 °C, the IDU solution was decanted, and after washing in 2 or 3 changes of Hanks' solution, the cells were inoculated with the virus. Cultures not treated with IDU were similarly infected to serve as control.

Determination of buoyant density: The virus material was centrifuged in CsCl solution of 1.45 and 1.32 g/ml density, in an MSE Superspeed 50 ultracentrifuge, for 4 h at 28,000 r.p.m. (100,000 *g*), at +9 °C temperature. The centrifuge tube was punctured for collection of the fractions, which were then examined for refraction coefficient in an Abbe-type refractometer.

Electron microscope examinations. The fractions obtained by ultracentrifugation were examined in a JEM 100 B JEOL electron microscope, after negative counter-staining with 1% uranyl acetate or 2% PTA.

Serological tests

Immune sera. Four rabbits and four guinea-pigs were immunized with the virus isolate by three i.v. plus i.m. injections at 5-day intervals, using inocula of 1, 2 and 3 ml, respectively. The animals were exsanguinated 19 days after the last immunization. Anti-hexon sera were prepared to hexones of different human adenovirus types (1, 8, 9, 10 and 12) (Nász et al., 1963). The anti-hexon sera were used for complement fixation (CF) and agar gel diffusion precipitation tests. Virus neutralization tests were performed with antisera to type 1—4 pig adenoviruses, kindly supplied by Dr. A. Bartha (Veterinary Research Institute, Budapest), and to type Ba 2, Ba 3 and Ba 6 bovine adenoviruses. A human adenovirus antiserum pool (9 types) and 34 monospecific equine adenovirus antisera were available by the courtesy of R. Wigand (Homburg, Saar, GFR) and J. G. Hierholzer (Atlanta, USA).

Agar gel diffusion precipitation tests were performed in 3-mm thick agar plates or layers, as described by one of us (Bodon, 1965).

Detection of a common CF antigen was attempted with human sera as described by Nász et al. (1963).

Neutralization tests. The neutralizing antibody titre of hyperimmune rabbit and guinea pig sera was determined in primary rabbit kidney cell cultures, against 1000 $TCID_{50}$ virus, using twofold serial dilutions of serum and a constant virus dose. Rabbit and equine antisera to porcine, bovine and human adenoviruses were similarly tested, except for dilution in tenfold serial steps.

Serum samples were collected from young rabbits at the onset of disease at 5—8 weeks of age, and one month later, as well from finished broiler rabbits weighing 2.5—3 kg at slaughter in the abattoir.

The mixtures of virus dilution and serum were incubated for 1 h at 37 °C, then 0.2 ml of each was transferred to primary rabbit kidney cell culture

grown in a Leighton tube. Before inoculation the growth medium of the cultures was exchanged to maintenance medium. Five to seven days after infection the coverslip cultures were removed from the Leighton tubes, stained with acridine orange, and read. Untreated cell cultures and cultures treated with virus only were stained simultaneously for use as control. Evaluation was based on the presence or absence of Cowdry type-A intranuclear inclusions.

Results

Experimental observations on healthy (group A), slightly ill (group B) and dying or dead rabbits (group C) are shown in Table I.

Table I

E. coli counts in various intestinal segments of healthy, slightly ill and severely ill or dead rabbits aged 6—8 weeks

		Group A Healthy rabbits	Group B Rabbits in initial stage of diarrhoea	Group C Rabbits in final stage of diarrhoea or carcases
Caecal content dry matter %	$\bar{x}$	26	12	8.5
	s	0.8	1.2	0.6
	n	12	15	15
Stomach	$\bar{x}$	0	0	0
E. coli	s	0	0	0
$\log_{10}$/g	n	12	15	15
Ileum	$\bar{x}$	1.5	2.3	7.8
E. coli	s	0.9	1.4	1.0
$\log_{10}$/g	n	12	15	15
Caecum	$\bar{x}$	2.7	4.7	8.2
E. coli	s	1.4	2.2	0.7
$\log_{10}$/g	n	12	15	15
Ileum oocysts/field of vision (×400)	$\bar{x}$	1.5	1.2	1.0
	s	0.6	0.9	0.7
	n	12	15	15

The proportion of dry matter was about 26% in the caecal contents of healthy rabbits, with little individual variation, and the *E. coli* counts of the chymus were generally low, 10^3/g in the ileum and 10^1—10^4/g in the caecum. Against this the severely ill or dead rabbits showed a marked dilution of intestinal contents (8.5% dry matter) and considerably increased caecal and ileal *E. coli* counts ($10^{7.8}$—$10^{8.28}$/g). Rabbits in the initial stage of disease, with a slight diarrhoea, had watery intestinal contents, but their intestinal *E. coli* counts increased scarcely over normal.

The causal responsibility of intestinal coccidiosis for the diarrhoea could be ruled out with certainty, for animals of all three groups showed practically identical, low oocyst counts in smears of intestinal mucosa, irrespective of their clinical condition.

In virus isolation experiments the primary rabbit kidney cell cultures showed neither gross nor microscopic signs of CPE in the first six blind passages of infectious material. The inoculated cultures were observed for seven

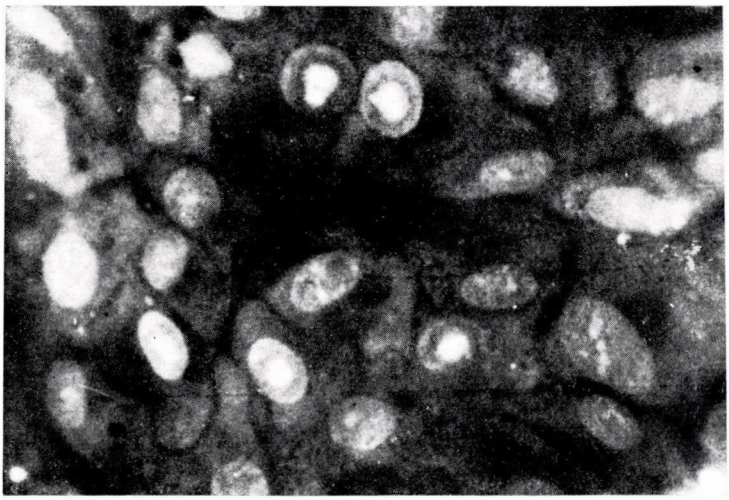

Fig. 1. Cowdry type-A intranuclear inclusions in a primary rabbit kidney epithelial cell culture infected with the rabbit adenovirus isolate. Acridine orange staining, ×640

days after each transfer. Before discarding the 6th passage as negative, we stained the cultures with acridine orange, which revealed the presence of Cowdry type-A intranuclear inclusions in a few cells. In these the nuclear membrane was thickened, and was separated by an unstained halo from the round, centrally placed inclusion body, which showed a greenish fluorescence in the UV-light. In the further passages intranuclear inclusions already appeared 3—5 days after infection (Fig. 1). The cell monolayer remained preserved despite the presence of intranuclear inclusions.

Intranuclear inclusions were induced by all organ suspensions prepared from each of the 7 rabbits tested. Their earliest appearance was observed in those inoculated with suspension of intestinal wall and intestinal contents. The initial titre of the virus isolate was 10^{-5}. In further passages the virus titre rose to 10^{-8}. The virus isolate was carried through 6—10 blind passages in several heterologous cell cultures, but it failed to replicate in these.

The isolate proved to be resistant to trypsin, Tween 80, pH 3.0 and heat.

Both methods used for nucleic acid typing unequivocally indicated that the isolate was a DNA virus. The intranuclear inclusions showed the green

fluorescence characteristic of acridine orange-stained DNA and no virus synthesis took place in the cultures treated with IDU.

The fractions collected after ultracentrifugation for 4 h were, after determination of their refraction coefficient, processed for electronmicroscopic examination by negative counterstaining. The fraction of 1.32 g/ml density contained icosahedral particles 80 nm in diameter, resembling the virion of Adenovirus. Similar particle did not appear in any other fraction.

The hyperimmune sera prepared with the isolate in rabbits and guinea-pigs had neutralization titres of 1 : 64 — 1 : 128 and 1 : 256 — 1 : 512, respectively. Antisera to porcine and bovine adenoviruses failed to neutralize the isolate. Equine antisera prepared with types 1—35 of human adenoviruses (except type 30, which had not been available) had no neutralizing effect either. It should be noted that the majority (60%) of the undiluted anti-human-adenovirus equine sera proved to by cytotoxic for the primary rabbit kidney cell culture.

Neutralization tests with sera from 5—7 weeks old rabbits suffering from diarrhoea revealed a low antibody level in only 10 of the 20 samples tested. One month later 3 of the 10 rabbits devoid of antibody died. Another month later the sera of the remaining 17 rabbits were tested again for neutralizing antibodies. By this time all animals had acquired antibodies in the titre range of 1 : 10—1 : 100. Seven to nine weeks old and older (slaughtery) rabbits had for the most part a titre of 1 : 100.

CF tests performed with hexon antisera to the human adenovirus types 1, 8, 9, 10 and 12 revealed presence in the isolate of a common CF antigen at 1 : 40—1 : 80 titre.

Immunodiffusion tests performed with the same materials indicated a partial antigenic identity (spur formation) of the rabbit isolate with the human adenovirus types tested.

Experimental infection of four to five week old susceptible rabbits reared in the laboratory with a total dose of 1 ml (10^5 $TCID_{50}$) virus material by intranasal plus subcutaneous route failed to induce a clinical illness.

Discussion

Authors generally agree that rabbit diarrhoea is due to excess multiplication of intestinal *E. coli* bacteria, whose toxic products elicit diarrhoea, followed by exsiccosis and sometimes by death of the animal. Our own observations did not substantiate this explanation. Field studies in a rabbit production unit with a history of recurrent diarrhoea among weanlings showed that the exalted *E. coli* counts reported in the literature occurred only in terminal illness or postmortem state (group C) while in the initial stage of the disease

(group B) the intestinal contents were watery (12% dry matter), but contained little more *E. coli* bacteria than normally ($10^{2.3}$—$10^{4.7}$/g), or even showed no increase thereof at all over normal counts (group A). Dilution of the intestinal content and consequent diarrhoea obviously preceded the excess multiplication of *E. coli*, which thus cannot be regarded as the primary causative agent.

It follows that factors other than *E. coli* toxin can also account for dilution of the caecal content, and exaltation of the *E. coli* count, as found in severely ill and dead rabbits, is only a sequel to the diarrhoeic condition.

Intestinal coccidia may well account for primary diarrhoea, as demonstrated by LÖLIGER et al. (1969), but their causal role could be ruled out with certainty in the flock studied by us.

This led us to consider the probable aetiological involvement of a virus (viruses?), and to perform virus isolation experiments in the early stage of the diarrhoea, when the *E. coli* counts were still low.

Isolation of an adenovirus from the intestines and other organs of rabbits with incipient diarrhoea have supported the hypothesis that a primary virus infection might give rise to a disturbance in caecal water metabolism which, through depression of the anti-bacterial resistance mechanisms, favours the exaltation of intestinal *E. coli* bacteria secondarily.

The adenovirus was isolated chiefly from the intestinal wall and chymus of 5—7 weeks old weanling rabbits; some animals had a low level of specific serum antibodies at this stage, but most carcases had none. The antibody levels were generally higher (1 : 10—1 : 100) at 7—9 weeks of age and later. At slaughter in the abattoir, antibody titres were found exclusively around 1 : 100. It should be noted that no mass incidence of diarrhoea has occurred in rabbit flocks older than 7—9 weeks.

The supposed aetiological involvement of adenoviruses in rabbit diarrhoea is supported by analogous observations in other species. Virus strains (corona-, rota- or reo-like viruses) isolated from pigs and calves suffering from diarrhoea proved to be neither sole agents of the disease, nor severely pathogenic, but there were certain indications that the primary virus infection had favoured the excess multiplication of the *E. coli* strains responsible for aggravation of the condition (MCADARAGH et al., 1971; RITCHIE et al., 1971; MENGELING et al., 1972; STAIR et al., 1972). DOUGALL and PETERS (1974) have regarded the avian adenoviruses only as "contaminants", but have postulated their involvement as synergists in the pathogenesis of *E. coli* septicaemias.

Our rabbit adenovirus isolate has some remarkable features. It showed the general electron-microscopic properties and some biological properties of adenoviruses but, unlike other adenoviruses, which adapt themselves readily to various heterologous tissues, it failed to replicate in various non-rabbit cells including the HeLa cell line. This and the lack of its neutralizability by porcine

and bovine adenovirus antisera, as well as by equine antisera to human adenoviruses have unequivocally shown that it represents not so much a rabbit-borne strain of some mammalian or human adenovirus as a genuine rabbit adenovirus. We have therefore requested the International Commission on Virus Nomenclature to agree that our rabbit adenovirus isolate be designated as Ra 1.

Summary

An adenovirus strain was isolated from the intestinal wall, intestinal contents and visceral organs of diarrhoeic rabbits in a large rabbit production unit noted for recurrent mass incidence of the condition in the weanling flock. Isolation was successful in the early stage of the disease, when the intestinal content had turned watery, but the intestinal *E. coli* count was still low. The isolate could be propagated exclusively in rabbit cell cultures, it failed to replicate in various heterologous cells generally used for the propagation of other adenoviruses. In primary rabbit kidney epithelial cell culture the isolate gave rise to Cowdry type-A intranuclear inclusions observable in stained preparations. No other grossly or microscopically visible CPE made appearance in the first passages. The isolate was identified as a DNA virus. It proved to be resistant to chloroform, pH 3.0 and heat. Its sedimentation rate was 1.32 g/ml and the virion diameter was 80 nm. It was neutralizable with specific hyperimmune sera prepared with the isolate in rabbits and guinea pigs, but antisera to porcine, bovine and human adenoviruses equally failed to neutralize it.

In CF tests performed with the isolate and with anti-hexon sera to certain human adenoviruses the common CF antigen was demonstrated in the titre range of 1 : 40—1 : 80. Partial antigenic identity was signified by spur formation in agar gel diffusion precipitation tests.

The 5—7 weeks old weanling rabbits involved by diarrhoea had low serum antibody levels, if any. Survivors already showed medium to high (1 : 10—1 : 100) titres, and older animals generally possessed high (1 : 100) levels of serum antibody. The possible pathogenetic importance of adenoviruses in rabbit diarrhoea is discussed.

The rabbit adenovirus isolate being the first ever reported in the world literature. The authors propose to the International Commission on Virus Nomenclature to classify the virus into the family *Adenoviridae* under the designation RA 1, or Adenovirus r 1.

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PROPERTIES OF THE "CKp" STRAIN OF TRANSMISSIBLE GASTROENTERITIS VIRUS

II. STUDIES ON THE *IN VIVO* VIRAL REPLICATION IN NEWBORN PIGLETS

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An immunofluorescence (IF) assay on the *in vitro* viral replication of the CKp strain of transmissible gastroenteritis (TGE) virus was previously reported (MOCSÁRI, 1976). This paper is a report on the *in vivo* replication that has been followed up in newborn piglets. The following questions were studied

— does the CKp vaccine virus replicate in the small intestine of piglets when orally given, as the virulent TGE virus does?

— if so, does it affect the columnar epithelial cells of the small intestine?

Materials and methods

A dose of $10^{6.81}$ $TCID_{50}$ of the CKp TGE vaccine virus (CSONTOS and SZENT-IVÁNYI, 1971) was given orally to 16 neonatal piglets of two TGE-negative sows within 4 h after birth. The piglets were nursed by the TGE-negative sows. Three or four pigs were exsanguinated at 24, 48, 72, 96 and 120 h after exposure.

The replication of the vaccine virus was followed up by immunofluorescence (IF) in the small intestine and, occasionally, also in the parenchymatous organs (spleen, liver, kidneys and lung), tonsils and mesenteric lymph nodes. The small intestines of the piglets that had been exsanguinated at 24, 72 and 96 h after exposure were also examined by light- and electron microscopy. Re-isolation of the vaccine virus was attempted from the small intestines.

Immunofluorescence (IF) assays. Approximately 2-cm segments were collected from the duodenum, jejunum and ileum as indicated in Fig. 1. The segments were placed on a filter paper and opened lengthwise. The mucosa was gently rinsed and lightly scraped with a scalpel. The scraping was placed on a precleaned microscopic slide and spread to a uniform smear by using another slide. Other organs were embedded in Cryoform (Damon, IEC Division, USA) and 2 to 3 μm thick cryostat sections were prepared. The intestinal smears and cryostat sections were fixed in a mixture of 1 volume of methanol

and 3 volumes of acetone for 10 min at room temperature. The fixed preparations were stained with a 1 : 64 or 1 : 128 dilution of a TGE conjugate for one hour at 37 °C, rinsed three times (total of 15 min) in phosphate-buffered saline (PBS) and in distilled water. The FITC conjugate was prepared from the colostral IgA fraction of an SPF sow that had been exposed to virulent TGE virus (MOCSÁRI and STONE, 1978). The counterstaining with Evan's blue

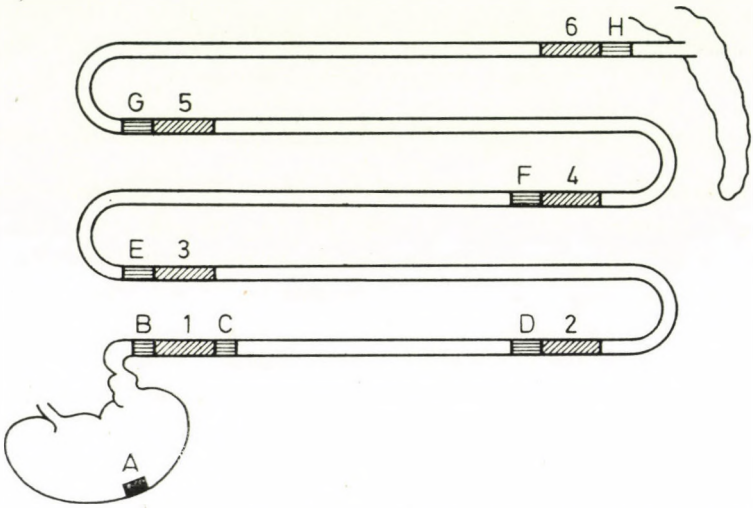

Fig. 1. Intestinal segments assayed by immunofluorescence (IF) and electron microscopy (EM) ▨ IF; ▤ EM

and the mounting procedure were previously reported (MOCSÁRI, 1977). The stained smears and sections were examined with an incident-light fluorescence microscope (Fluoval, Zeiss) using a 200-W mercury vapour lamp, BG-12 exciter filter and OG-4 barrier filter.

Light and electron microscopy (EM). Immediately after the exposed piglets were exsanguinated, the small intestine was removed and 1-cm segments were taken from the areas indicated in Fig. 1. The segments were fixed in 2.5% phosphate-buffered glutaraldehyde at pH 7.2 and postfixed in Millonig's osmium tetraoxide at pH 7.2, dehydrated in graded alcohols and embedded in Durcupan. The semithin sections for light microscopy were stained with toluidine blue. The ultrathin sections, stained with uranyl acetate and lead citrate, were examined by a Philips 201 CS electron microscope.

Attempts to re-isolate the vaccine virus. Stationary cultures of secondary swine thyroid (Sth) cells were prepared in 400 ml Roux flasks. The mucosa from different segments of the small intestine was removed by scraping. The mucoid epithelium was resuspended to 10% in Hanks' balanced salt solution (HBSS) containing 100 units of penicillin G, 100 μg streptomycin, 50 μg

gentamycin sulphate and 25 units of Nystatin per ml, as well as 0.5% lactalbumin hydrolysate (LAH) and 15% bovine serum. The volume of the inocula was 2 ml. Following a one-hour adsorption period at 37 °C, the inoculum was removed, the cultures were washed twice with PBS and 30 ml HBSS containing 0.5% LAH and 1% bovine serum was added. The cultures were daily examined for cytopathic effects (CPE). Coverslip cultures of secondary Sth cells that had been prepared in Leighton tubes were inoculated with the medium of the cell cultures immediately after the appearance of the CPE or following two consecutive blind passages. Eighteen hours post inoculation, the coverslip cultures were removed and examined for TGE viral antigen by IF.

Results

The results of IF tests and the attempts to re-isolate the vaccine virus are shown in Table I.

In the intestine of piglets exsanguinated 24 to 72 h after exposure, the specific fluorescing viral antigen was mainly observed in the cytoplasm of individual villous epithelial cells (Fig. 2) or in small clusters of the adjacent epithelial cells (Fig. 3). In the 96th after exposure, many more fluorescing cells appeared in the intestinal smears, forming continuous layers of fluorescing cells (Fig. 4). In the lung sections, fluorescing viral antigen was mainly observed in the cytoplasm of individual alveolar epithelial cells or in small

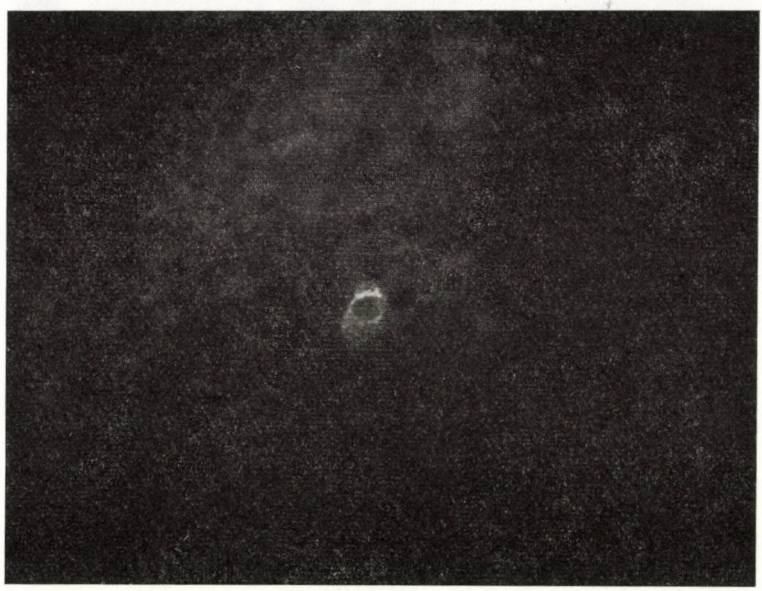

Fig. 2. IF 24 h after exposure. Specific fluorescing TGE viral antigen in the cytoplasm of a single columnar epithelial cell in the jejunum. ×600

Table I

The results of IF assays and attempts to re-isolate the vaccine virus

Organs	Exsanguinated after 24 hours			after 48 hours			
	A	B	C	A	B	C	D
Results of IF assays							
Spleen	NT	NT	—	NT	NT	—	—
Liver	NT	NT	—	NT	NT	—	—
Kidney	NT	NT	—	NT	NT	—	—
Lung	NT	NT	—	NT	NT	—	+
Tonsils	NT	NT	—	NT	NT	—	—
Small intestine							
1	—	—	++	—	—	—	—
2	—	—	—	—	—	—	+
3	—	—	—	—	—	+	++
4	—	—	—	—	—	++++	++
5	—	—	—	—	—	+++	—
6	—	—	—	—	—	++	—
Mesenteric lymph nodes	NT	NT	—	NT	NT	—	—
Virus re-isolation from the intestine	—	—	POS	—	—	POS	POS

Explanations. A—D, marking of pigs; 1—6, segments from the small intestine (see Fig. 1); epithelial cells; ++ in a number of individual epithelial cells; +++ in small clusters of the

Fig. 3. IF 48 h after exposure. Specific fluorescing viral antigen in the cytoplasm of a number of adjacent epithelial cells (jejunum). ×600

in 16 neonatal piglets exsanguinated 24 to 120 h after vaccination

72 hours			96 hours			120 hours		
vaccination								
A	B	C	A	B	C	A	B	C
NT	NT	—	NT	NT	—	NT	NT	—
NT	NT	—	NT	NT	—	NT	NT	—
NT	NT	—	NT	NT	—	NT	NT	—
NT	NT	++	NT	NT	++	NT	NT	—
NT	NT	—	NT	NT	++	NT	NT	—
—	+	—	—	+	—	+	++	—
—	+	—	+++	+++	—	++	—	—
—	—	—	++++	++++	++	+	—	—
+	—	—	++++	++++	++	+	—	—
—	+	—	+++	++++	++	++	+	+
+	+	+	++++	+++	+++	++	+	+++
NT	NT	—	NT	NT	—	NT	NT	—
—	—	—	POS	POS	POS	POS	—	POS

NT, not tested; POS, positive; — negative. Specific fluorescing TGE viral antigen: + in a few adjacent epithelial cells; ++++ in larger continuous layers of the adjacent epithelial cells.

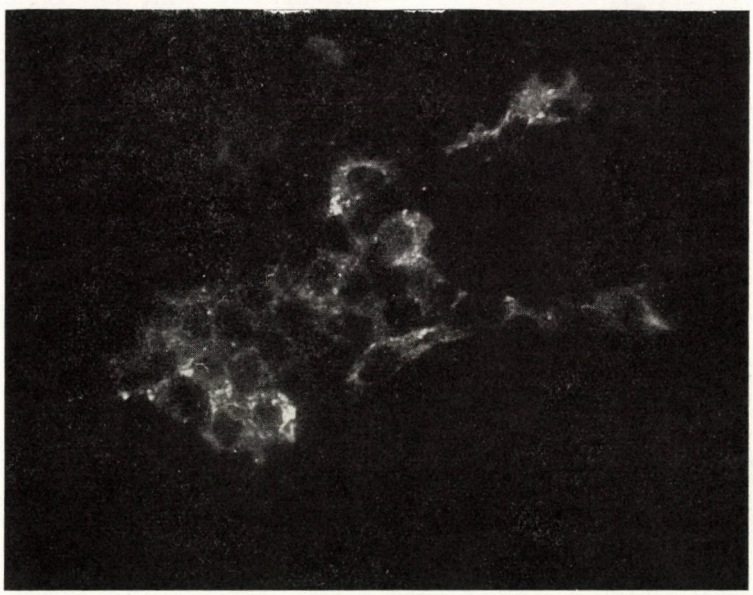

Fig. 4. IF 96 h after exposure. Clusters of columnar epithelial cells in the ileum, filled with bright-fluorescing viral antigen. ×600

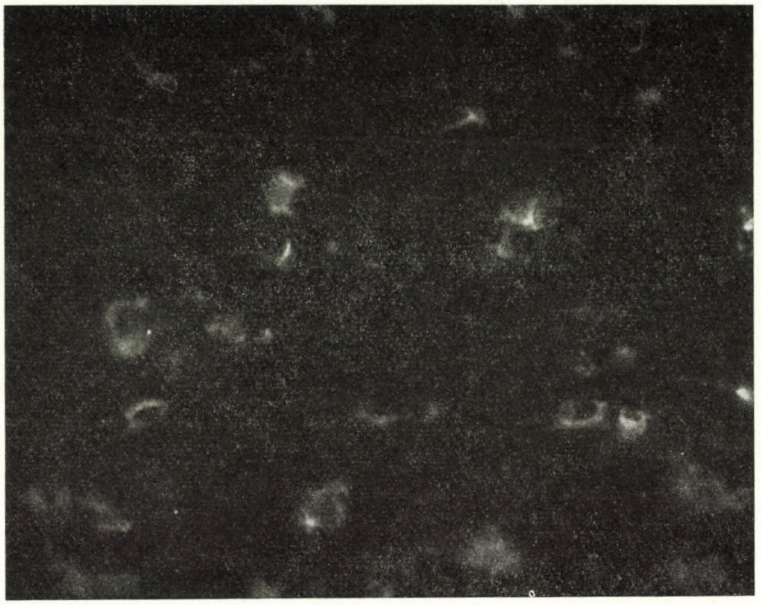

Fig. 5. IF 96 h after exposure. Specific fluorescing alveolar epithelial cells in the lung. ×600

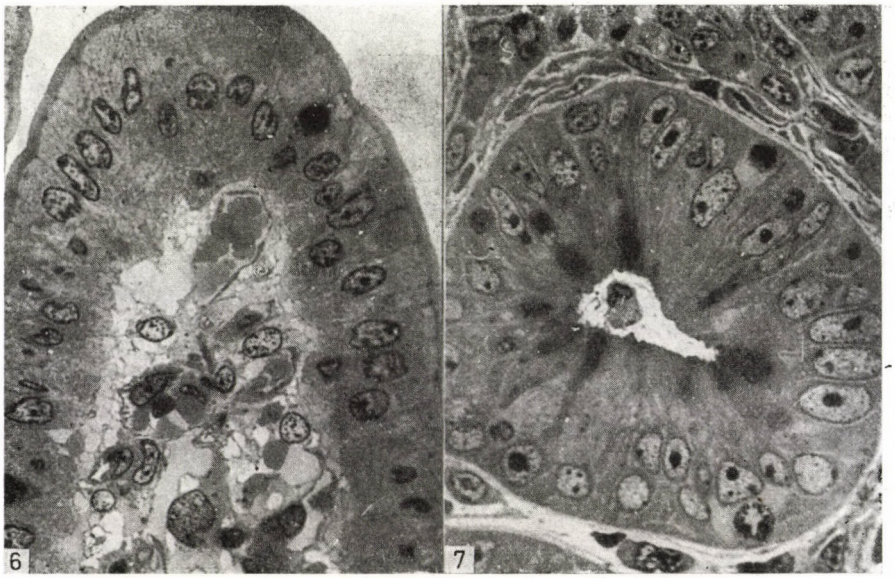

Fig. 6. Transversal section of a jejunal villus from a pig 96 h after exposure. The columnar epithelial cells are intact. Semithin section, toluidine blue stain. ×890

Fig. 7. Cross section of a crypt from a pig 96 h after exposure. No histological lesions can be observed. Semithin section, toluidine blue stain. ×1600

Fig. 8. Electron micrograph of a villous absorptive cell in the jejunum from a pig 96 h after exposure. Viral particles with an average diameter of 90 nm (arrows) in the cytoplasm, within cytoplasmic vacuoles. Uranyl acetate—lead citrate. ×77,900

clusters of the adjacent cells (Fig. 5). TGE viral antigen was also observed in the tonsils from one of the piglets exsanguinated 96 h after exposure, in the germinative centres. Viral antigen could not be demonstrated in any of the other organs that had been investigated.

No histological lesions were observed either in the intestinal villous or in the crypt epithelial cells (Figs 6 and 7). By EM, viral particles with an average diameter of 90 nm were found in the cytoplasm of the differentiated intestinal epithelial cells, often within a cytoplasmic vacuole (Figs 8 and 9).

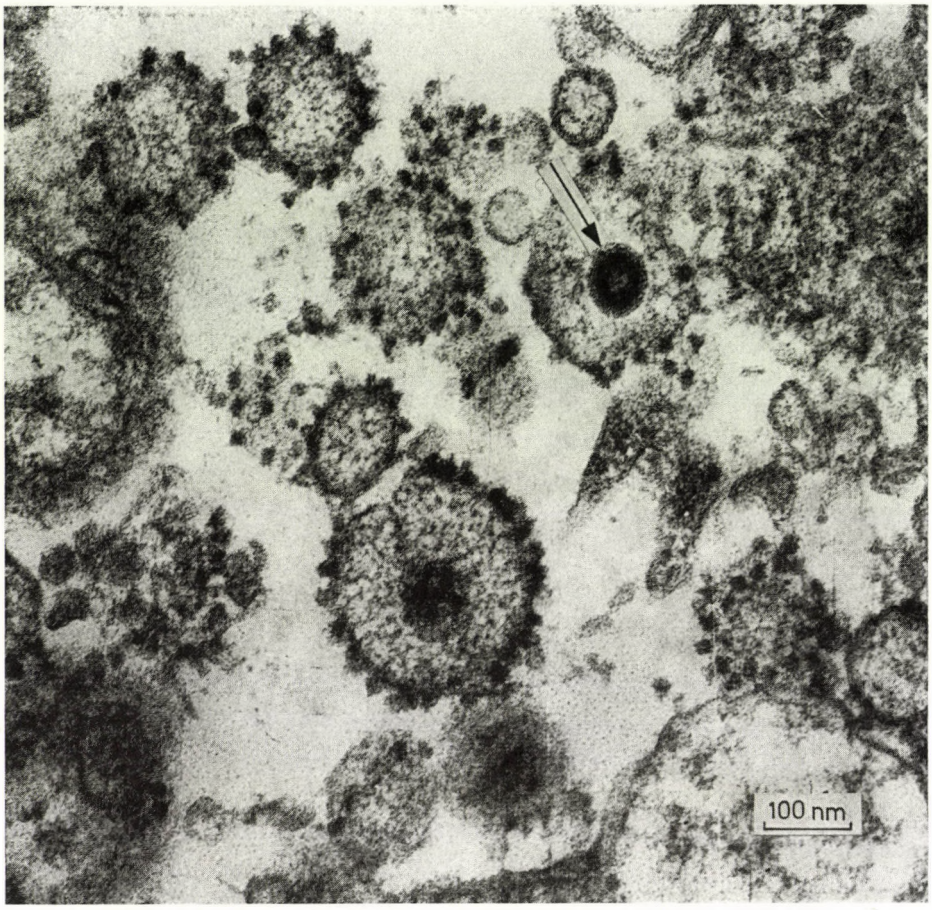

Fig. 9. The same as in Fig. 8. A single enveloped viral particle in the lumen of the granular endoplasmic reticulum. Uranyl acetate—lead citrate. ×120,000

Discussion

It is a well-established fact that the mechanisms of the immunity in TGE differs from those in most of the known infectious diseases. The differences come from the pathogenesis of TGE and are connected with the special affinity of TGE virus for columnar epithelial cells of the small intestine. The principal importance of both the active and the passive immunity is to protect the villous epithelial cells of the small intestine from TGE viral infections.

The active immunity is provided by the protection or resistance of an appropriate number of villous epithelial cells against TGE virus. The local elaboration of the secretory IgA-type antibodies and their secretion on the

mucosa surface, as well as the cellular immunity might play equal roles in the basic mechanisms of the active immunity (CARTWRIGHT, 1967; FREDERICK and BOHL, 1976; SPRINO et al., 1976). The term "lactogenic immunity" is used to describe the mechanisms of the passive immunity transmitted from immune sows to offspring (HAELTERMAN and HOOPER, 1967). Suckling pigs are protected as a result of the frequent ingestion of colostrum and milk containing TGE virus-neutralizing antibodies. Such antibodies in the lumen of the gastrointestinal tract will tend to neutralize any ingested TGE virus before the virus can infect the susceptible columnar epithelial cells of the small intestine (HAELTERMAN and HUTCHINGS, 1956; HOOPER and HAELTERMAN, 1966; HAELTERMAN and HOOPER, 1967; BOHL et al., 1972). The most effective protection is provided by the continuous secretion of the IgA-type antibodies in the milk. A significant level of TGE antibodies of the IgA class has been demonstrated in the colostrum and milk only after an infection of the gastrointestinal tract (BOHL et al., 1972a, 1972b).

With this information, it is obvious that an effective immunity to TGE can only be achieved by oral vaccination if the vaccine virus is capable of replicating in the columnar epithelial cells of the small intestine. Adaptation to cell culture, however, could lead to the failure of the attenuated TGE virus to infect the intestinal epithelial cells. After a given number of cell culture-passages, varying strain by strain, the attenuated TGE virus can completely or particularly lose the ability to replicate in the columnar epithelial cells and the viral replication is restricted to the caudal portion of the small intestine. Consequently, in spite of the oral vaccination with an attenuated live virus, the columnar epithelial cells remained in their entirety or in majority susceptible to virulent TGE viral infections. Such animals will produce no, or only low levels of TGE antibodies of the IgA class in the milk, due to the failure or to only a mild antigenic stimulation of the gastrointestinal tract (FREDERICK et al., 1976; HESS et al., 1977).

The criteria of an ideal strain of TGE vaccine virus has been formulated by BOHL (cit. PENSAERT, 1976): the vaccine virus should be still "virulent" enough to replicate in the epithelial cells of the small intestine and stimulate the production of TGE antibodies of the IgA class but attenuated to such a degree not to cause any or only a mild diarrhoea even in susceptible neonatal pigs.

Studies carried out so far have provided information that the CKp strain of TGE virus meets in every respect the criteria of the ideal TGE vaccine virus. In a series of laboratory experiments, CSONTOS and SZENT-IVÁNYI (1971) have established the innocuity of the vaccine virus in a reliable manner. A great number of field trials have also proved the innocuity of the vaccine virus (CSONTOS et al., 1974; BENYEDA et al., 1974; MOCSÁRI et al., 1975a, 1975b; MOCSÁRI et al., 1976; MOCSÁRI, 1978). From the favourable experiences

that had been obtained with this vaccine during field vaccination trials and TGE outbreaks, it could also be suggested that the vaccine virus replicated in the columnar epithelial cells of the small intestine as the virulent TGE virus did. Also the immunoglobulin characteristics of the antibodies in the milk of the vaccinated sows support this suggestion *viz.*, in orally vaccinated sows, similarly to the sows recovered from natural infection, the specific virus-neutralizing activity was associated primarily with IgA (MOCSÁRI, 1980).

The follow-up of the *in vivo* viral replication has verified the reliableness of this hypothesis. The results of the IF assays and EM examinations as well as the re-isolation of the vaccine virus from the small intestine have proved that the CKp TGE virus retained the ability to replicate in the intestinal villous epithelial cells during the attenuation in cell culture. The vaccine virus is still "virulent" enough to replicate in the columnar epithelial cells along the small intestine (see Table I), providing an effective protection for the vaccinees to the entire length of the intestine against virulent TGE viral infections. Besides, the replication of the vaccine virus, in contrast to that of the virulent TGE virus, causes no detectable microscopic lesions in the intestinal epithelial cells.

Summary

The *in vivo* replication of the CKp strain of TGE vaccine virus was followed up in neonatal piglets by direct immunofluorescence (IF). Besides, the small intestine was examined by light- and electron microscopy.

The IF studies revealed that the vaccine virus replicated primarily in the columnar epithelial cells of the small intestine and also in the lung and tonsils. Viral replication could be demonstrated along the small intestine. The vaccine virus was also demonstrated by EM in the cytoplasm of the differentiated epithelial cells in the small intestine. Re-isolation in cell cultures was successful from the small intestinal samples.

By light microscopy, no histological lesions were observed either in the intestinal villous or in the crypt epithelial cells.

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LT ANTITOXIN TITRES IN THREE-WEEK-OLD, *E. COLI* -VACCINATED PIGS

By

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Previous studies have shown that sera of sows and pigs as early as 8 weeks of age might possess antibody-neutralizing activity against *E. coli* heat-labile (LT) enterotoxin (GYLES, 1974; WILSON et al., 1971). Furthermore, it has been revealed that in sera of pigs the amount of LT-neutralizing antibodies rose gradually with progressing age (WHIPP and DONTA, 1976; PESTI and SEMJÉN, 1978). After natural infection with enteropathogenic strains of *E. coli* an elevation of anti-LT titres of sera was demonstrated in humans (SACK et al., 1974). Vaccination of sows with a complex, inactivated, enterotoxin-containing *E. coli* vaccine resulted in a notable rise of LT antibody levels in their sera and colostrum (PESTI and SEMJÉN, 1976).

E. coli diarrhoea has been frequently observed among pigs weaned at approx. 28 days of age (SEMJÉN and SZABÓ, 1976).

In the available literature there are no data whether LT-antibodies can be detected in sera of piglets aged 22—32 days and whether vaccine preparation injected at this age brings about elevation in anti-LT titre. In the present study we compared the anti-LT titre of 22-day-old pigs' sera taken before, and 10 days after, vaccination.

Materials and methods

From 24 piglets, 22 days of age, kept on a commercial ration in a large pig farm, blood samples were taken. Out of them, 14 animals were simultaneously vaccinated, 10 uninoculated pigs served as control. Two ml vaccine was injected i.m. The complex vaccine consisted of formol-inactivated LT and ST enterotoxins and killed bacterial cells. The pigs were weaned at 28 days of age. When the animals reached the age of 32 days, the blood sampling was repeated in both groups. The slightly modificated ligated rabbit intestinal loop test of KASAI and BURROWS (1966) was used for measuring the LT antitoxin titres. The antitoxin titre was expressed as antitoxin units per ml of serum.

Results and discussion

All the serum samples derived from 22-day-old pigs contained heat-labile enterotoxin-neutralizing antibodies. The antitoxic titres ranged from 16.8 to 85.8 units per ml.

The antitoxic titres measured in sera of 32-day-old pigs, which had been weaned 4 days earlier, were from 18.3 to 66.0 units per ml.

The mean titres for the two groups were 42.9 and 33.5 units per ml, respectively (Table I). Sera obtained from nonvaccinated, 32-day-old pigs had a somewhat lower mean titre, but the decrease proved not to be significant.

Table I

Antitoxin titres to *E. coli* heat-labile enterotoxin in sera from 22- and 32-day-old pigs with and without *E. coli* vaccination

Group	Age		
	22 days	32 days	
		Vaccinated*	Non-vaccinated
No. of pigs	24	14	10
Antitoxic titers, $\bar{x} \pm SE$	42.86 ± 4.43	138.77 ± 16.36**	33.54 ± 4.46

* Pigs were vaccinated at 22 days of age; ** highly significant *vs.* the two other groups.

No information is available in the literature on the level of LT-neutralizing antibodies in sera of pigs at age of 22 or 32 days. GYLES (1974) examined 8-week-old pigs.

The antibodies passively acquired *via* the colostrum decline in a very short period of time.

During the second and third week of life of pigs, a low or negligible level of antibodies against the somatic O and K antigens of *E. coli* was found (SHARPE, 1965; PORTER and HILL, 1970). The serum level of IgM increases after the 1st week of life, while the IgG and IgA levels show a continuous diminution for more than 3 weeks (PORTER and HILL, 1970).

Thus, there are two possible explanations for the origin of LT-neutralizing antibodies found in sera of 22-day-old pigs: 1. these antibodies might be remnants of colostral antibodies, or 2. they may be formed by the piglets themselves supposedly as a consequence of an earlier contamination and/or infection with LT-producing strains of *E. coli*.

For the lack of knowledge on the type of immunoglobulins which carry the specific LT-neutralizing activity, however, no reliable conclusion can be drawn as regards to their origin.

Vaccination of piglets at age of 22 days increased significantly the LT antibody level (Table I). The average of titres in vaccinated animals was four times as high as that of the non-vaccinated group and reached the value which had been found in adult sows (PESTI and SEMJÉN, 1976).

Recently accumulated data on cholera disease strongly supported the fact that parenteral immunization with an appropriate vaccine gave rise to

immunity and protection against subsequent challenge with cholera toxin or live vibrios (HOLMGREN et al., 1977; FINKELSTEIN, 1970; SVENNERHOLM and HOLMGREN, 1977; HOLMGREN et al., 1975).

Thus, it was reasonable to assume that parenteral immunization with the complex *E. coli* vaccine also could help for the protection against the post-weaning *E. coli* diarrhoea of pigs. On the basis of experiments presented here, however, it could not be ascertained whether the significant elevation of circulating antibodies might contribute to the local defence mechanism. Further experiments are necessary to check the reliability of our hypothesis.

Summary

Parenteral immunization of 22-day-old pigs with complex, formol-inactivated *E. coli* vaccine resulted in significant, approximately fourfold, rise of LT antitoxin levels in pigs' sera as determined by the method of KASAI and BURROWS (1966).

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THE DEVELOPMENT OF PRIMARY ANTIBACTERIAL IMMUNE PROTECTION AGAINST *ERYSIPELOTHRIX RHUSIOPATHIAE* AND THE EFFECT OF ANAEROBIC CORYNEBACTERIAL IMMUNOSTIMULANTS

(SHORT COMMUNICATION)

By

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The immunomodulating effect of *Corynebacterium parvum* and its extracts on microbial immunity has been studied recently by FAUVE (1975), CERUTTI (1975), KNIGHT and LUCKEN (1975) as well as by our team (RÉTHY et al., 1978). Using *Erysipelothrix rhusiopathiae* vaccine as test-antigen, we investigated the following problems.

To what extent corynebacterial whole cell suspensions influence the development of primary antibacterial immunity if immunomodulation is initiated

(i) prior to the administration of immunizing agent,

(ii) simultaneously with the test vaccine, or

(iii) following immunization.

As immunopotentiators, merthiolate-inactivated corynebacterial whole-cell suspensions were used. The strains from which vaccines were prepared are listed in Table I. The individual dose per mouse corresponded to 2.0 Opacity Units (O.U.) as adjusted against the International Opacity Standard (N.I.H., Bethesda, USA) in 0.2 ml amounts subcutaneously. *E. rhusiopathiae* vaccine contained 3×10^9 inactivated bacterial cells and 5 mg of aluminiumhydroxide adsorbent per ml. Swiss/Phylaxia albino mice-groups were immunized on one occasion with individual doses of 0.2 ml of each of twofold serial dilution-series of *E. rhusiopathiae* vaccine. The challenge was performed with 1000 LD of virulent *E. rhusiopathiae* culture on the 14th day following immunization. On the basis of the PD_{50} values found in differently treated groups and expressed in ml of the test vaccine, the protective effect of different immunization procedures was given in P.U. $\pm$ SD (Protective Units per ml) and R.P. (relative potency as compared to the effectiveness of the vaccine in the untreated control group).

The computation was done on the basis of REED and MUENCH's method (1938) as modified by PIZZI (1950).

On the basis of data given in Table I the following facts can be stated.

(i) *Corynebacterium* immunopotentiators given 3 days prior to the immunization with the test-antigen did not affect the development of primary antibacterial immunity.

Table I

Timing and effect of *Corynebacterium* immunopotentiators' administration on the development of primary anti-*Erysipelothrix rhusiopathiae* immunity

Treatment on day*	−3			0			+3		
C. strains**	PU±SD		RP	PU±SD		RP	PU±SD		RP
C. parvum RR-1	80	3.79	0.74	159.6	5.45	1.47	31.5	2.54	0.29
C. parvum RR-12	126	4.41	1.16	176	6.71	1.62	60.6	3.88	0.55
C. avidum RR-41	93.5	3.85	0.86	233.8	7.29	2.15	34.8	6.58	0.32
C. acnes RR-2	126	4.83	1.16	165.5	4.93	1.52	101.1	4.35	0.93
C. lymphophylum RR-33	80	3.91	0.74	168.14	5.65	1.55	80	4.62	0.74
C. granulosum RR-H5	92.4	4.16	0.85	156.8	5.03	1.35	92.4	3.99	0.85
Control vaccine***				108.7	5.32	1.0			

Explanation: * related to the day of immunization with the test-vaccine (0-day); ** the taxonomic names of strains are given according to PRÉVOT et al. (1967); *** no immunopotentiator-treatment.

(ii) Immunopotentiators given simultaneously with the test vaccine increased the potency of the *E. rhusiopathiae* vaccine 1.35—2.15 times. The increase of the potency, as compared to the results achieved in the control group is statistically significant.

(iii) Corynebacterial immunostimulants given 3 days following immunization exhibited immunosuppressive activities in cases of treatments with *C. parvum* RR-1, *C. parvum* RR-12 and *C. avidum* RR-41 immunostimulant suspensions. In groups treated with *C. acnes* RR-2, *C. lymphophylum* RR-33 and *C. granulosum* RR-H5 immunopotentiators, however, no change was observed in the expression of test-vaccine's potency as compared to the result got in the control group.

The results got in groups with prior administration of corynebacterial immunopotentiators suggest a rapid, but short-lived, activation of cells involved in the immune reaction. In the case of the simultaneous administration of corynebacterial suspensions and the test-vaccine, this rapid activation of the immune system would appear to coincide with the priming activity of the introduction of the antigen. The immunosuppressive activity of some of the strains used when supplied after the immunization may be explained by the

two major characteristics of *Corynebacteria* when used as immunostimulants on the basis of results published by HANAZAWA et al. (1978), *viz.* (i) the proliferation-inducing activity is exerted mainly at the level of macrophages, which of course includes the proliferation of splenic suppressor cells, too, and (ii) the *Corynebacterium* cells are bound to the plasma membrane of macrophages.

In view of afore mentioned characteristics of corynebacterial immunostimulants (immunomudulants) the test antigen-induced proliferation of cells involved in the immune sequence may be qualitatively and/or quantitatively altered by the timing of the administration of the immunopotentiators.

It may be concluded that the immunomodulating effect of corynebacterial immunostimulants exerted on the primary antibacterial (anti-*E. rhusiopathiae)* immunity depends on the timing of its application.

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HEAT-INACTIVATED *BRUCELLA* SUSPENSIONS AS MODULATORS OF HOSTS' DEFENCE AGAINST TUMOUR

(SHORT COMMUNICATION)

By

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One of the main achievements of the past decades' immunological research is the discovery of the possibility of manipulating the immunological-type homoiostatic regulation. For these purposes, among others, immuno-modulants of bacterial origin have extensively been administered. In a series of our recent publications, we comparatively tested the immuno-modulating activity of immunostimulants prepared from anaerobic *Corynebacteria* in primary antitoxic antibacterial and tumour models (GÉRESI et al., 1980; PADÁNYI et al., 1980; RÉTHY et al., 1978).

In our present paper we describe the results achieved in a screening-type experiment aimed at defining the antitumour activity of heat-inactivated *Brucella* suspensions. The immunostimulation induced by heat-killed *Brucella abortus* organisms is known from the works published by ROUMIANTZEFF et al. (1974) and TOUJAS et al. (1972, 1974a).

Brucella suspensions harvested from potato-agar surface cultures were heat-inactivated. The bacterium count was adjusted to 10.0 opacity units (O.U.) per ml against the International Opacity Standard. The single mouse dose amounted to 2.0 O.U. in 0.2 ml volumes. Mouse groups consisting of 20 mice (Swiss albino) each were formed at random. All the mice were inoculated with 2.5×10^5 viable Ehrlich ascites tumor cells i.p. on the "0" day of the experiment. Mice exhibiting no signs of ascites on the 7th day following tumour-introduction were excluded from the experiment. The immunomodulator treatments were started on the 7th day of the experiment. Groups A received immunopotentiator suspensions corresponding on the whole to 8.0 O.U. subcutaneously in four separate doses at four days intervals. Groups B were given daily doses of brucella suspensions for a ten-day period, *i.e.* 20.0 O. U. altogether, subcutaneously.

Mean Survival Time (MST$\pm S_{\bar{x}}$) was calculated. The statistical analysis (t test) was made on the basis of differences between MST values. The figure $>CMST\%$ represents the percentage of animals in test groups surviving the MST period of untreated controls.

On the basis of the data collected in Table I the following facts can be stated.

Table I

Antitumour activity of immunostimulants prepared from different *Brucella* strains

Immunostimulants		MST$\pm S_{\bar{x}}$ (days)	>CMST % survival	P *vs.* control
origin*	dose in Σ O.U.			
Brucella abortus	8	34.3± 7.7	40.0	n.s.
PH-0048	20	39.55± 8.3	56.0	<0.1
Brucella abortus	8	56.2± 8.7	78.0	~0.002
PH-0159	20	56.1±10.6	75.0	0.001
Brucella abortus	8	34.8± 5.0	44.4	n.s.
PH-0260	20	49.2± 9.4	60.0	~0.02
Brucella abortus	8	54.1±10.9	56.0	0.01
PH-371	20	32.7± 7.7	30.0	n.s.
Brucella suis	8	37.0±10.2	40.0	n.s.
PH-482	20	35.9± 6.57	50.0	n.s.
Untreated controls		29.4± 3.5	20.0	

* Strain-numbers given according to the PHYLAXIA register; n.s., not significant.

1. Using brucella-originating immunomodulator no correlation was found between dose and prolongation of survivals.

2. Except the immunomodulator prepared from *B. suis*, the administered brucella suspensions did increase the MST value in treated groups. Statistically significant increase was found after treatments with *B. abortus* strains no. PH-0159., PH-0260., and PH-0371. The prolongation of survivals caused by treatment with immunomodulator vaccine prepared from *B. abortus* strain no. PH-0048 may be regarded as tendency only ($P<0.1$). *B. suis* strain no. PH-0482 exhibited no antitumour activity.

3. Regarding the data concerning the percentage of treated animals surviving the MST of untreated controls, we found that the prolongation of survivals was caused by the individual and treatment-specific prolongation of lifespans in the majority (56—78%) of the animals.

4. Animals surviving the acute phase lost clinical and tumour-specific symptoms. The main characteristic of the remission phase was the disappearance of the ascites. Finally, the animals died of metastatic isolated tumours.

In conclusion, we demonstrated that there are *B. abortus* strains exhibiting in an inactivated form, major antitumour activity in a nonisogenic animal-tumour model. The antitumour effect does not seem to exhibit a par excellence dose-dependence. The antitumour activity of immunomodulator is not yet clear in spite of the explanations of HALL and MOORE (1974) and TOUJAS et al. (1973, 1974b) who claimed that the immunomodulator activity of *B. abortus*-originating immunomodulator would exert a direct effect on the lymphoid cells and macrophages. The above-described results cannot give any evidence as to the action-mechanism of antitumour activity of the investigated immunomodulator. The question needs further investigations.

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ANTITUMOUR EFFECT OF DIFFERENT CORYNEBACTERIAL IMMUNOSTIMULANTS IN NON ISOGENIC MOUSE-TUMOUR-SYSTEM

(SHORT COMMUNICATION)

By

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It has been well established that certain microorganisms or bacterial extracts increase the resistance to a variety of unrelated bacterial or viral infections and stimulate immunity against tumours in animals and man by amplifying the immune potential. Recent results in the field of tumour-immunity stressed corynebacterial immunostimulants as modulators of non-specific immunity (HALPERN, 1974; HALPERN et al., 1964; PADÁNYI et al., 1980; RÉTHY et al., 1978, 1980; REGELSON, 1976; SUIT et al., 1978; WOODRUFF, 1964, 1974; WOODRUFF et al., 1972, 1976).

Using Ehrlich's ascites tumour in non-isogenic mice, we investigated the anti-tumour effect in a screening type experiment of immunostimulants prepared of different strains of anaerobic *Corynebacterium*.

The anaerobic cultures of *Corynebacteria* (listed in Table I) were centrifuged, resuspended and merthiolate-inactivated. The density of bacterial suspensions was adjusted against the International Opacity Standard and given in Opacity Units (O.U.) per ml. Mouse groups (Swiss albino), bred and selected at random, consisted of 20 mice each. All the mice were inoculated with 2.5×10^5 viable Ehrlich's ascites tumour cells, i.p. on the "0" day of the experiment. (Mice showing no apparent ascites on the 7th day following tumour introduction were excluded from the experiment.)

The immunostimulant treatments were started on the 7th day of the experiment with a subcutaneous dose corresponding to 2.0 O.U. in 0.2 ml volume. Groups A received 2 O.U. on each of four successive days, whereas Groups B were given daily doses for a ten-day period, *i.e.* 20.0 O.U.-s altogether.

The Mean Survival Time ($MST \pm S_{\bar{x}}$) was calculated. The statistical analysis was made on the basis of MST values with the t test. The figure >CMST%

Table I

Antitumour activity of different corynebacterial immunostimulants

Immunostimulants		MST$\pm S_{\bar{x}}$ (days)	>CMST % survival	P *vs.* control
origin*	dose in Σ O.U.			
C. parvum RR-1	8	40.6±5.57	43.3	~0.01
	20	28.5±3.24	20.0	—
C. parvum RR-12	8	46.1±6.3	71.4	0.001
	20	38.8±6.55	50.0	(<0.1)
C. acnes RR-2	8	45.7±9.6	60.0	~0.01
	20	25.3±2.85	10.0	—
C. avidum RR-41	8	39.1±7.25	70.0	<0.05
	20	40.9±8.53	60.0	<0.05
C. lymphophylum RR-33	8	28.5±3.12	30.0	—
	20	41.1±8.99	44.4	<0.05
C. granulosum RR-H5	8	37.3±6.72	57.1	(~0.1)
	20	22.5±3.98	10.0	—
Untreated controls		29.4±3.5	20.0	

* Taxonomic names given as in: PADÁNYI et al. 1980; P., 8.0 O.U. *vs* 20.0 O.U.~2.

represents the percentage of animals in treated groups surviving the MST of the untreated controls.

On the basis of results collected in Table I the following statements can be made.

1. The effect of immunostimulant treatment in general seems to be dose-dependent. Each of *C. parvum, C. acnes* and *C. granulosum* immunostimulants exhibited an antitumour effect of higher degree when applied in lower doses. Immunostimulant bacterial suspension prepared of *C. lymphophylum,* on the contrary, was found more powerful in higher doses, the *C. avidum*-originating immunostimulant, however, exhibited antitumour activity of the same degree in both doses (p% 8→20 O.U.~2).

2. All the immunostimulant vaccines did, or tended to, increase the MST in the treated groups. Statistically significant increases were registered with the both of the *C. parvum* preparations as well as with the *C. acnes, C. avidum* and *C. lymphophylum* immunostimulant suspensions. In other cases a tendency-type activity of immunostimulants can be supposed only.

3. An analysis of the data concerning the percentage of survivals above the MST value of the control group, has proved the specific antitumour effect

of immunostimulant vaccines. The prolongation of the MST in treated groups was caused by the individual and treatment-specific prolongation of the lifespan in the majority (44—71.4%) of the animals.

4. Animals surviving the MST of the untreated controls developed ascites as a rule in the acute phase. As a consequence of the immunostimulant treatment the ascites disappeared. The animals died, after a prolonged lifespan, in the exacerbation phase. Postmortem analysis showed metastatic type, solid and isolated tumours.

In conclusion, we have proved that the antitumour activity of investigated anaerobic corynebacterial immunostimulants shows a rough correlation with the dose and number of introductions of immunostimulants. The Ehrlich's ascites tumour responds well to treatments with immunostimulants of corynebacterial origin in nonisogenic host-tumour model.

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THE EFFECT OF ANAEROBIC CORYNEBACTERIAL IMMUNOSTIMULANTS ON THE PRIMARY IMMUNE RESPONSE TO *TETANUS* TOXOID AND *PERFRINGENS* EPSILON TOXOID

(SHORT COMMUNICATION)

By

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The effect of *Corynebacterium parvum* inactivated suspensions on the development of primary humoral immunity had been studied by GALL (1972) and KNIGHT and LUCKEN (1974). GALL investigated the immune response against diphtheria toxoid while KNIGHT and LUCKEN defined the immunostimulant effect on the development of active protection against tetanus intoxication. These authors observed no immunostimulant activity while administering *C. parvum* suspensions.

In recent publications (GÉRESI et al., 1980; PADÁNYI et al., 1980; RÉTHY et al., 1978, 1980) we described a relatively wide variety of immunological properties of immunostimulants prepared from a series of corynebacteria and brucellae.

In the present paper we describe results concerning the effect of corynebacterial immunostimulants on the primary immune response (primary type immune protection against intoxication) to tetanus toxoid and perfringens epsilon toxoid.

Antigens: Serial dilutions were made of tetanus toxoid (PTAP., 1.0 m = 12.0 B.U. = 50 I.I.U.) and perfringens epsilon toxoid adsorbed on aluminium hydroxide (1 ml = 30,000 MLD-equivalent = 429 P.U.). Groups of Swiss albino mice were formed at random, each consisting of 20 animals. The immunizing dose of the antigens corresponded to 0.5 ml of each dilution. The individual doses of the immunostimulant vaccines corresponded to 2.0 O.U. (Opacity Units) mixed with the immunizing antigens. The mixture of antigen and immunostimulant was applied subcutaneously. The preparation and standardization of corynebacterial immunostimulants is described in our recent paper (PADÁNYI et al., 1980). The immunized animals were challenged

on the 21st day following immunization with 20 LD_{50} of tetanus toxin or 3 MLD of perfringens epsilon toxin. On the basis of proportion of survivors in control and immunostimulant-treated groups we calculated the dose required to protect 50% of the animals: PD_{50}. Using the PD_{50} figures, the relative potency (R.P.) of immunization procedures and the virtual protective

Table I

The development of primary antitoxic protection against tetanus toxin and perfringens epsilon toxin and the effect of corynebacterial immunostimulants

Immunostimulants origin*	Degree of primary antitoxic protection against			
	tetanus toxin		perfringens epsilon toxin	
	I.I.U./ml***	R.P.**	P.U./ml****	R.P.**
C. parvum RR-1	73.0	1.46 (1.29—1.65)	948	2.21 (2.0 —2.43)
C. parvum RR-12	37.5	0.75 (0.66—0.85)	214.5	0.5 (0.57—0.54)
C. acnes RR-2	43.0	0.86 (0.76—0.97)	n.c.	0.5 n.c.
C. lymphophylum RR-33	91.0	1.82 (1.52—2.18)	429.0	1.0 (0.93—1.08)
C. avidum RR-41	68.0	1.36 (1.13—1.63)	n.c.	0.5 n.c.
C. granulosum RR-H5	64.0	1.28 (1.11—1.47)	214.5	0.5 (0.41—0.61)
No immunostimulant	50.0	1.00 (0.88—1.13)	429.0	1.00 (0.82—1.22)

* Taxonomic names given as in: PADÁNYI et al. (1980); ** R.P., Relative potency (95% fiducial limits); *** I.I.U., International Immunizing Unit (WHO Standard); **** P.U., Protective Units (FAO Standard, Weibridge).

effect expressed in I.I.U. and P.U. (International Immunizing Unit, Protective Unit) were calculated. The 50% effects were computed according to KAERBER's method with stating the 95% fiducial limits.

The results of the experiment are presented Table I. On the basis of the results the following statements can be made.

1. Effects of *Corynebacteria* immunostimulants on the development of primary anti-tetanus immunity were defined as changes in the development of primary-type active protection against tetanus toxin intoxication.

Significant increases in active protection were recorded in cases of joint application of tetanus toxoid and immunostimulants prepared of *C. parvum* RR-1 [I.I.U. = 75., R.P.: 1.46 (1.29—1.65)] and *C. lymphophylum* RR-33 [I.I.U. = 91., R.P.: 1.82 (1.52—2.18)]. Tendency-type increases of primary antitetanus immunity were found in mice given *C. avidum* RR-41 and *C. granulosum* RR-H5 inactivated suspensions. *C. parvum* RR-12 caused significant suppression of the development of immunity, while in case of *C. acnes* RR-2 the suppression of immunity was of tendency-type only.

2. The joint effect of perfringens epsilon toxoid and corynebacterial immunostimulants caused immunosuppression in the majority of the cases.

C. lymphophylum RR-33 had no effect on the development of antitoxic-type protection, while *C. parvum* RR-1 increased the degree of protection [P.U. = 948., R.P.: 2.21 (2.0—2.43)].

In conclusion, we can state that our results concerning the effect of corynebacterial immunostimulants on the development of antitoxic immunity (antitoxic protection) impair with the results published by GALL and KNIGHT and LUCKEN. The results unanimously show that the corynebacterial immunostimulants generally increase the degree of protection against tetanus in case of joint application of immunostimulant with tetanus toxoid. KNIGHT and LUCKEN in their experiment applied the immunostimulant intravenously and subcutaneously. Their "immunostimulant" suspension was prepared of *C. parvum*. It is interesting that our *C. parvum* RR-12 strain also depressed the protection against tetanus.

Among the corynebacterium strains investigated by us, only the *C. parvum* RR-1 exhibited an adjuvant effect on the development of primary antiperfringens epsilon immunity. The other strains had immunosuppressive properties or, as in case of *C. lymphophylum* RR-33, no effect.

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STORAGE-RELATED POTENCY CHANGES IN MONOVALENT TYPE O_1, A_5 AND C FOOT-AND-MOUTH DISEASE VACCINE PREPARATIONS

By

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Foot and mouth disease (FMD) vaccines tend to loose potency after a given time. Instructions for use of commercial FMD vaccine preparations usually give the expiration time as one year. Practical observations have shown that certain preparations may retain their full potency longer. Expired batches were therefore occasionally re-tested for efficacy, and the expiration time was appropriately prolonged.

The influence of storage temperature on the efficacy of three Frenkel-type FMD vaccines was studied by Coudert et al. (1972). At +4 °C, the potency generally decreased slowly, but to different degrees depending on the type, the O, A and C type test vaccines remained potent for 40, 36 and 18 months, respectively. An O-type preparation showed no change in efficacy after 48 h storage periods at —10 and +45 °C, compared to a lot stored as usual at +4 °C. However, storage of the same preparation at —20 and +55 °C equally resulted in a significant decrease in potency. On checking by 50% bovine protection test the potency of BHK-produced FMD vaccines 1 to 2 years after their issue, Mowat (1974) found no loss of potency in PSF-1860 produced O-type vaccines after prolonged storage up to 2 years. The A- and C-type vaccines, prepared from the Uruguayan Pandó strain and the Noville strain, respectively, proved to be fully potent after one year. Similar observations were reported by Kucsera et al. (1977), who found that certain Frenkel-type and Waldmann-type vaccines remained potent for 2 to 3 years.

In the present study the storage-related decrease in potency was followed up in Waldmann-type O_1-, A_5- and C-type vaccines prepared from virus strains propagated *in vivo* in bovine tongue epithelium. The probable interrelationships of potency decrease with the pH, free formalin and antigen contents of the vaccines were also pursued.

Materials and methods

Vaccines

The potency of vaccines prepared in PHYLAXIA by Waldmann's method was checked at different times after production and issue. Each preparation contained aluminium hydroxide gel corresponding to 0.75% aluminium oxide and 4 mg saponin per dose as adjuvant. The recommended bovine

dose was 2 ml in each case. Sixteen, 11 and 25 batches of O_1, A_5 and C type monovalent vaccines, respectively, were regularly tested for efficacy over a 3 year period. A single large sample of each preparation, stored in a bottle at +4 °C, was used for testing throughout.

Quantitative complement fixation test (CF)

This test was employed for determination of the antigen content. Binding of standardized complement (1 C'HD_{50} = 7 µl) by the virus antigen was determined in the presence of antiserum giving maximum reaction, and the results were expressed in µl. The CF activity of the fluorcarbon-treated virus antigen was assessed as the quantity of complement bound by unit antigen and was expressed in terms of α GF.

Efficacy tests

Guinea pig tests. Guinea pigs of our own random breed, weighing 450 to 500 g, were used. Vaccine potency was tested by the C-index. method, *i.e.* one part of the animals was vaccinated, the other part served as control. Ten days after treatment a guinea pig-adapted homologous strain was titrated in both groups. The titre difference in logarithmic terms serves as the C index. Vaccine potency is satisfactory if the value of the C index is 2 or higher.

Adult mouse tests. Balb/c×CBA F_1 crosses, weighing 20 to 22 g, were used for testing with the E-index method. One group of the animals was treated parenterally with 1/10 bovine dose, and immunity was challenged 14 days later. The challenge strain was of the same type as the vaccine strain, but it had been adapted to adult mice. The unvaccinated control animals were treated with the challenge strain along with the vaccinees. The E index corresponds to the intergroup titre difference in logarithmic terms. Potency is adequate if the value of the E index is 2.39 or higher.

Results

The monovalent type O_1, A_5 and C vaccines were tested for potency one week after finishing, and at various times during storage. The longest time interval between the finishing of vaccines belonging to the same type was 2 months. At planning the experiment, it had to be decided whether each preparation should be tested exactly one month after finishing, or all preparations representing a given type may be tested simultaneously. The latter approach presupposed a not longer than 2-month time interval between the finishing dates. Simultaneous tests at type level were preferred to elimination

of the minor time differences by individual testing. Thus, preparations of the same type were tested simultaneously, by the mouse protection test, results of which are shown in Fig. 1. Mean values of the E index were calculated for

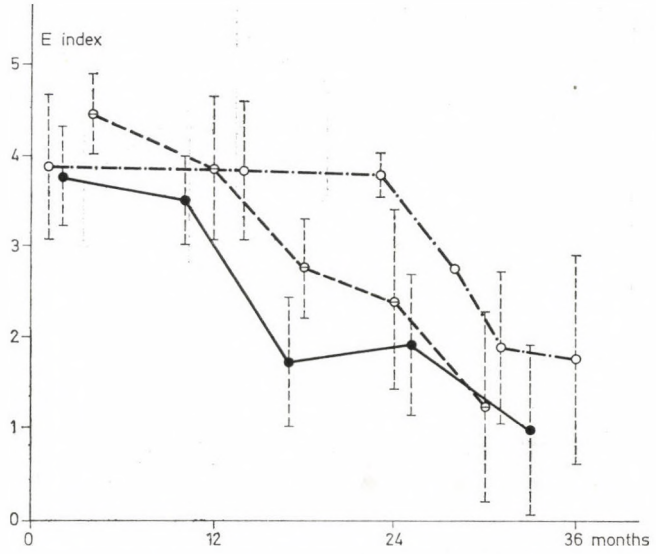

Fig. 1. Mean value and standard deviation of potency in type O_1, A_5 and C monovalent FMD (Waldmann) vaccines, as determined by the E-index method at different times after finishing. ⊖---⊖ O_1-type vaccine; ●——● A_5-type vaccine; ○-·-○ C-type vaccine

each vaccine type, along with the standard deviation. As can be seen, storage for one year caused no significant decrease in potency in any type tested, and type C even maintained a satisfactory potency for 2 years. The rate of potency loss was greatest in type A_5, lowest in type C and intermediate in type O_1. Testing of the type-A_5 vaccines on two additional occasions after drop of their potency below the permissible level showed that no further decrease occurred between one and 2 years of storage, but an abrupt drop followed after 2 years.

The results of the guinea-pig tests are shown in Fig. 2. Mean C-index values and their deviation were calculated for each vaccine type.

The length of the persistence of potency at the level set forth in the standard prescriptions was assessed by the two different tests as follows:

Test	Vaccine types		
	O_1	A_5	C
C-index method	23 months	20 months	32 months
E-index method	24 months	14 months	29 months

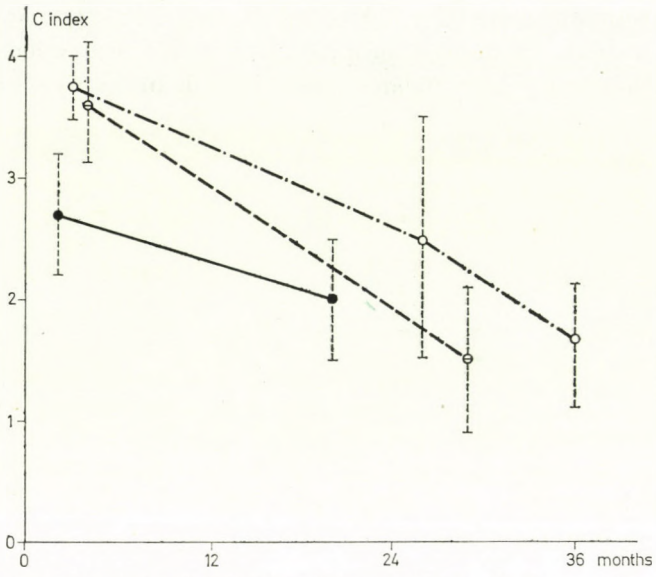

Fig. 2. Mean value and standard deviation of potency in type O_1, A_5 and C monovalent FMD (Waldmann) vaccines, as determined by the C-index method at different times after finishing. ⊖---⊖ O_1-type vaccine; ●——● A_5-type vaccine; ○— • —○ C-type vaccine

The maximum difference found between the results of the two tests was 3 to 6 months within the type category. The greatest difference was found in type A_5; according to our observations, deviation is the greater the lower the immunogenicity of the vaccine, and among the vaccine types tested, A_5 was in fact the least immunogenic. Both tests revealed the same sequence of potency loss: type A_5 vaccines maintained potency for the shortest time, type O_1 ones for longer and type C ones for the longest time. Figs 1 and 2 also show that the deviation of potency indices was generally minor at the beginning, but it tended to become greater with progressing time. Increased deviation was due to intra-typic variation, *i.e.* to variable duration of potency in vaccines of a given type. The following minimum and maximum durations of potency were found within the type categories tested:

Indices	Min-max times of potency loss Vaccine types		
	O_1	A_5	C
C index	16—30 months	14—35 months	20—28 months
E index	19—36 months	10—31 months	24—37 months

The mean values have unequivocally indicated which antigen type had lost its potency at the earliest, but the tests also disclosed considerable intra-typic variation. It follows that the mean expiration time characteristic of the

type is not conclusive of the actual duration of potency in single batches. *E.g.* the type A_5 vaccines were found to have an average expiration time of 1.5 years, but some were still fully potent after 31 to 35 months.

A probable correlation of the expiration time with the antigen content was also pursued. The antigen contents of the test preparations were determined in CF units as

390 α GF	in type O
232 α GF	in type A
169 α GF	in type C

The antigen contents were plotted versus expiration times (Fig. 3), and correlation coefficients were calculated for each type separately, and for all together. No correlation was found by either approach. While generally the C-type vaccines had the lowest antigen content in terms of CF units, exactly these preparations maintained their potency for the longest periods. Intratypic analysis in the categorie C also failed to reveal any impact of the quantity of antigen on the length of expiration time although freshly prepared vaccines abundantly containing antigen have been shown to be more potent than those

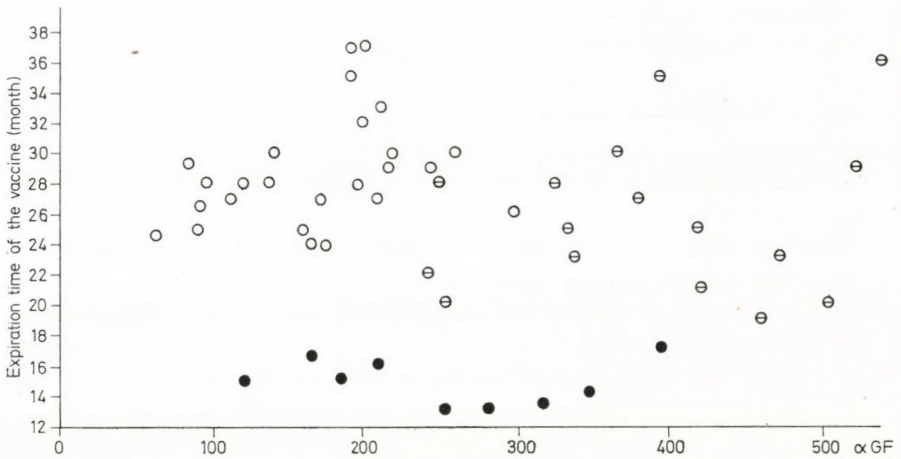

Fig. 3. Correlation between expiration time and antigen content (expressed in CF units) of type O_1, A_5 and C monovalent FMD (Waldmann) vaccines. ⊖ O_1-type vaccine; ● A_5-type vaccine; ○ C-type vaccine

with a lower antigen content (CZELLENG and SÓLYOM, 1977). It is known that the FMD virus types differ considerably in antigenicity; it follows that less antigen of the highly immunogenic type gives a more potent vaccine than quantitatively much more antigen of a type of poor antigenicity. This can explain the high index values obtained in the present study with the C-type

vaccines, although, these contained less antigen than the type O_1 or A_5 ones. The decline of vaccine potency probably depends primarily on the stability of the antigen. Great differences have been established (FONTAINE, 1975; ROWLANDS et al., 1972) between the stability of the FMD virus types and subtypes. The virions of certain strains possess a particularly fragile capsid, the vulnerability of which may be exalted by inactivation. Evidence has been presented that inadequate inactivating agents cause even the most immunogenic virus to loose antigenicity within a short time. A good inactivating agent should not only prevent the incorporation of any infections virus into the vaccine, but also provide for preservation of the antigen for the longest time possible. It has been shown that formalin used for inactivation in the present study, meets both criteria, and is particularly suitable for the inactivation of viruses with a fragile capsid. However, failure of formalin to inactivate some highly (about 1000 times) concentrated FMD virus has also been observed (ADAMOWICZ et al., 1977). It appears that in the future the inactivating agent of choice should be selected individually for each vaccine strain, for a good inactivating agent can prolong the expiration time of the vaccine by several months to years.

The probable influence of pH changes on the length of expiration time was studied, in the pH range from 8.0 to 8.4. No correlation was found.

The free formalin content of the vaccines was found to be 273, 294 and 268 μg/ml in O_1, A_5 and C-type preparations, respectively. Originally, 400 μg/ml formalin had been added to each preparation for inactivation. The free formalin concentration had no bearing either on the duration of potency.

All vaccines used in the present study had been prepared by Waldmann's procedure. In the meantime the production firm changed to the Frenkel technique, and the Frenkel-type FMD vaccines are now under test for duration of potency. The experimental monovalent C-type preparations tested up to now proved to be still potent after 18-month storage, although the mean E-index value fell from 4.5 to 3.55.

Summary

On a total 52 monovalent FMD vaccines of types O_1, A_5 and C, prepared according to Waldmann's procedure, were examined for duration of potency over a three-year long period of storage. The potency tests were carried out on guinea-pigs with the C-index method and on adult mice with the E-index method. The preparations were tested one week after finishing, and after different periods of storage, using the same sample throughout. The following expiration times were assessed with the two tests:

Indices	Vaccine type		
	O_1	A_5	C
C-index	23 months	20 months	32 months
E-index	24 months	14 months	29 months

No significant decrease occurred in the potency of any vaccine type after one-year storage; the type-C vaccines even were still satisfactorily potent after two years. Potency was declining in type-A_5 vaccines at the earliest, in type-O_1 ones later and in type-C ones latest; this sequence of expiration times was consistently emerging from the type averages, but intra-typic variations in persistence of potency were great (10 to 38 months). The antigen content of the vaccines as expressed in CF units, changes of vaccine pH within a given range, and free formalin levels did not show any correlation with the length of expiration times. In view of the present findings, the one-year expiration time usually given for Waldmann-type vaccines can be regarded as adequate, but re-checking of expired batches seems to be rewarding.

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EFFECTS OF ANTIBIOTICS ON THE IMMUNE SYSTEM OF ANIMALS

I. MOUSE EXPERIMENTS WITH THE LOW-VIRULENCE VR_2 STRAIN OF *ERYSIPELOTHRIX RHUSIOPATHIAE*

By

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(Received August 28, 1978)

During the last decades antibiotics have been very widely used in the veterinary practice not only as therapeutics, but also as growth stimulators. The problem of their possible influence on the immune system of animals has therefore increasingly come to the fore. A certain impact of antibiotics on immune reactions has been taken into consideration also with regard to immunity acquired by active immunization.

An ontoward influence of antibiotics can be expected above all at their simultaneous application with live vaccines containing avirulent or low-virulence bacteria.

To obtain further information on the problem, I used *Erysipelothrix rhusiopathiae* bacteria as a model for experimental approach. It is known that already the first antibiotic, penicillin, has had a dramatic effect on swine erysipelas (GREY, 1947; ULBERG, 1953; MOYNIHAN and STOWELL, 1954; DZYALENKO, 1962; POLITYNSKA-BANAS, 1965; FORRAY, 1967; AZECHI et al., 1971; KINJO and MACHIDA, 1973), and has been, therefore, very widely used for its therapy. It was, nevertheless, soon recognized that pigs cured from erysipelas by penicillin treatment did not become immune against the disease (ORLANDELLO, 1955, 1960; NARDELLI and DAL PRATO, 1960; KOVALENKO and TATARINCHEV, 1962; AZECHI et al., 1972).

Since the prevention of swine erysipelas by vaccination with avirulent or low-virulence strains has become a widespread practice, many authors have investigated the influence of antibiotic treatment on the development of vaccination-stimulated immunity, but no unequivocal conclusions have been drawn. Certain authors (NYSTRÖM, 1957; OSE et al., 1963) found no difference between the responses of mice, fed and not fed an antibiotic-containing diet at vaccination, to later challenge with a virulent strain, while others presented experimental evidence that feed medication with chlortetracycline (COOPER and ALLEN, 1959) as well as various parenterally administered antibiotics (chloramphenicol, penicillin, streptomycin) equally exerted a disadvantageous influence on response to vaccinations (KOVALENKO and TATARINCHEV, 1962).

The observations made on mice were later confirmed in pig experiments (WILLINGER and STÖCKL, 1961; STÖCKL and WILLINGER, 1962; KOVALENKO and TATARINCHEV, 1962; LYASHENKO, 1965; KONYAEV and KARACIK, 1968), and it was proposed (LYASHENKO, 1965; SHCHETKIN, 1970) that antibiotic administration by any route should by preferably avoided from two days before to 10 days after immunization against swine erysipelas with a live vaccine. Other authors (WILLINGER et al., 1961; KONYAEV et al., 1968) were of the same opinion, but recommended a shorter period for antibiotic withdrawal.

No information has emerged from the above studies on the interrelationship between the size of the inoculum and the antibiotic dose, nor on the

impact of the duration of antibiotic withdrawal on vaccination efficiency. These aspects were studied in the present experiments performed on mice with the *E. rhusiopathiae* low-virulence vaccine strain VR_2.

Materials and methods

Erysipelothrix rhusiopathiae *strains*

Challenge strain: *E. rhusiopathiae* OK 22, serotype 1a, a laboratory strain stored in freeze-dried state. Subcutaneous administration of 10^1 OK 22 cells kills 50% of mice 20 g in weight.

Vaccine strain: *E. rhusiopathiae* VR_2, a serotype 2a strain of reduced virulence (ISOPESCU and GHEORGHIU, 1965), which has been regularly used as swine erysipelas vaccine strain in several countries (Bulgaria, Czechoslovakia, GDR, Rumania, USSR, Viet-Nam). The strain, available in freeze-dried state, has a certain residual virulence, *viz.*, 10^6 cells of it kill 50% of mice 20 g in weight on subcutaneous administration.

Antibiotic: Retardillin (Biogal, Hungary), benzyl-penicillin-procaine.
Crystalline penicillin (standard).

The minimum inhibitory concentration (MIC) and *minimum bacteriocidal concentration* (MBC) of penicillin were determined *in vitro* by titration in tubes. Twofold serial dilutions of crystalline penicillin (range: 0.0025—2.56 IU/ml) were added to horse-meat broth containing 10% inactivated horse serum. Then the media were inoculated with 24 h cultures of *E. rhusiopathiae*, using about 10^4 cells per tube as inoculum. The cultures were incubated for 24 h at 37 °C, and the titration endpoint (the highest dilution permitting bacterial growth) was read by visual inspection. Subsequently, inocula of each dilution step were transferred to solid agar medium, and incubated for 48 h at 37 °C, to determine the minimum bactericidal concentration.

In vivo determinations of MIC and MBC were carried out in mice. Appropriate groups of mice were injected subcutaneously with tenfold serial dilutions (range: 2×10^2—2×10^5) of bacterium suspension, and simultaneously in another site with rising doses of Retardillin (range: 1×10^4—8×10^4 IU), increased at twofold serial steps. The animals were observed for 14 days, and the results were evaluated on the basis of mortality rate and prolongation of the mean survival time.

The effect of Retardillin on immune response elicited by the VR_2 vaccine was studied in mouse experiments. Albino mice weighing 21.0 ± 3.1 g were injected in groups of 10 with different doses (2×10^3, 2×10^4 and 2×10^5 germs) of the VR_2 vaccine, and with either different doses of the antibiotic, or with an equal dose (40,000 IU/kg bwt), but at different times relative to vaccine administration. After 14-day observation the mice were infected with about

1000 LD_{50} of virulent OK 22 virus. Observation was continued for further 10 days, and losses were recorded. The heart blood of the died mice was spread on agar medium to recover the *E. rhusiopathiae* bacteria.

Two control groups, one untreated and the other immunized but not treated with the antibiotic, were set up with each test. Losses and survival among the mice treated with vaccine+antibiotic were expressed in per cent of the loss in the vaccine control. Each experiment was performed in three replica series.

Mean and standard deviation of the results were calculated for each experimental series, and inter-group differences were analysed for significance with Student's t test.

Results and discussion

First the strains OK 22 and VR_2 were compared for penicillin sensitivity. Analysis of the experimental results obtained *in vitro* revealed no notable difference. The MIC of penicillin was 0.04 IU/ml, and its MBC was 0.09 and 0.10 IU/ml for the strains OK 22 and VR_2, respectively.

The results of the *in vivo* experiments are shown in Tables I and II. The protective effect of penicillin depended greatly on the size of the vaccine

Table I

In vivo bacteriostatic and bactericidal action of Retardillin against the virulent *E. rhusiopathiae* strain OK 22 in mice

Infectious dose (number of living cells) / Retardillin dose (IU/kg bwt)	No. of mice infected	$2 \cdot 10^2$			$2 \cdot 10^3$			$2 \cdot 10^4$		
		Percentage of losses		Prolongation of survival time (hours)*	Percentage of losses		Prolongation of survival time (hours)*	Percentage of losses		Prolongation of survival time (hours)*
Untreated	30	10	100	113.77	10	100	92.17	10	100	90.60
10,000 IU/kg	30	2	20	—	10	100	134.95	10	100	104.17
20,000 IU/kg	30	0	0	—	8	80	137.95	10	100	109.29
40,000 IU/kg	30	0	0	—	4	40	—	9	90	123.79
80,000 IU/kg	30	0	0	—	1	10	—	8	80	136.15

* Harmonic mean.

dose with both the virulent (OK 22) and low-virulence strain (VR_2), and on the applied concentration of penicillin as well. Rising doses of the antibiotic conferred a correspondingly greater protection on the mice infected with a given bacterium dose. Conversely, rising infectious doses accounted for a corresponding increase in percentual losses if the antibiotic dose was kept constant.

Table II

In vivo bacteriostatic and bactericidal action of Retardillin against the *E. rhusiopathiae* vaccine strain in mice

Vaccine dose (number of living cells) / Retardillin dose (IU/kg bwt)	No. of mice vaccinated	Total no. of mice died	$2 \cdot 10^3$		$2 \cdot 10^4$		$2 \cdot 10^5$	
			No.	%	No.	%	No.	%
			of mice died		of mice died		of mice died	
Treated with vaccine only (vaccine control)	90	14	1	3.33±5.77	3	10.00±10.00	10	33.33±15.28
10,000 IU/kg	90	12	1	3.33±5.77	2	6.67± 5.77	9	30.00±10.00
20,000 IU/kg	90	12	1	3.33±5.77	1	3.33± 5.77	8	23.33± 5.77
40,000 IU/kg	90	6	0	0	1	3.33± 5.77	5	16.67± 5.77
80,000 IU/kg	90	2	0	0	1	3.33± 5.77	1	3.33± 5.77

The mouse-immunizing potency of different doses of the vaccine was established before analysis of the effect of Retardillin on immune response. The animals were immunized with different doses of vaccine, and were challenged to establish the percentage of protection. It was found that the immunogenicity of the vaccine greatly depended on its dose. While 10^5 bacteria per dose conferred a nearly 100% immunity, 10^3 bacteria protected scarcely more than 10% of the vaccinees against the challenge.

Table III

Immunogenicity of different doses of the swine erysipelas vaccine VR_2 in mice

Vaccine dose (number of living cells)	No. of mice		Protected animals, per cent ($\bar{x}\pm s$)	Relative inter-group difference
	vaccinated	challenged		
Unvaccinated (control)	30	30	0	—
$2 \cdot 10^2$	30	29	10.37±10.02	$P>0.05$
$2 \cdot 10^3$	30	29	44.81± 5.01	$P<0.01$
$2 \cdot 10^4$	30	29	82.59± 6.51	$P<0.01$
$2 \cdot 10^5$	30	25	96.67± 5.77	$P<0.05$

The relationship between vaccine dose and the immunity elicited by it can be characterized by the function

$$y = a \log x + b,$$

where x is the vaccine dose in terms of the number of living cells introduced in the vaccine, y the immunity in terms of percentual survival, a represents

the slope (tg a) and b the intersection of the straight line and the ordinate. The values a and b can be calculated from the experimental results, yielding the regression equation

$$y = 29.67 \log x - 54.12 \text{ (see Fig. 1).}$$

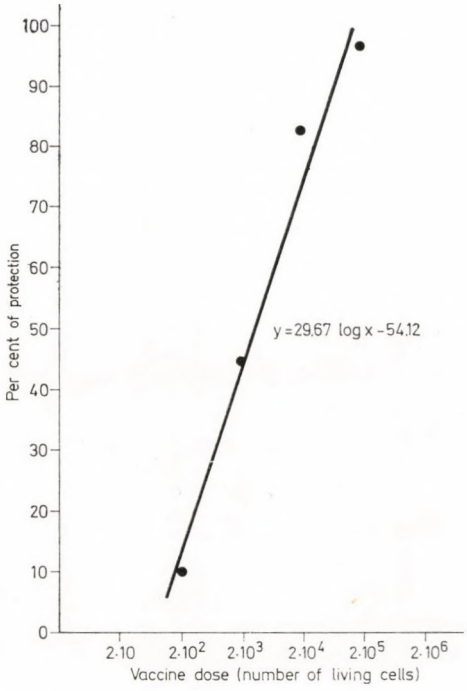


Fig. 1. Relationship between the dose and protective efficiency of the VR_2 vaccine

It follows that the protective potency of the vaccine VR_2, expressed as the percentage of surviving vaccinees, shows a positive linear regression with the logarithm of bacterium content in the dose of vaccine. Increase of the latter in the mouse model is, however, delimited by the residual virulence of the vaccine strain VR_2, which accounts for rising losses at elevated dose levels. The fact nevertheless remains that immune response to vaccination with the VR_2 strain of reduced virulence depends on the applied dose of the vaccine.

In possession of the above findings, I examined how administration of rising doses of Retardillin simultaneously with vaccination would influence the response of vaccinees to challenge.

The results obtained at three levels of treatment (see "Materials and Methods") are shown in Table IV and Figs 2 and 3.

Table IV

Effect of different Retardillin doses on immune response of mice to the vaccine VR_2

Retardillin dose (IU/kg bwt) \ Vaccine dose (number of living cells)	No. of vaccinated mice	No. of challenged mice	Mean protective efficiency ($\bar{x}\pm s$) $2 \cdot 10^3$ Protection (%)	P^*	$2 \cdot 10^4$ Protection (%)	P^*	$2 \cdot 10^5$ Protection (%)	P^*
Untreated	90	76	46.67±11.55	—	83.33± 5.77	—	100.00± 0.00	—
10,000 IU/kg	90	78	24.44± 7.70	<0.05	74.05±13.17	>0.05	90.00±10.00	>0.05
20,000 IU/kg	90	80	20.00±10.00	<0.05	50.95± 8.61	<0.01	86.90± 1.03	<0.001
40,000 IU/kg	90	84	10.74±11.13	<0.05	43.33± 5.77	<0.01	67.31± 7.40	<0.01
80,000 IU/kg	90	88	3.33± 5.77	<0.01	37.48± 3.85	<0.001	55.19± 5.01	<0.001

* Relative to vaccine control.

Table V

Influence of the time of Retardillin treatment on the immune response of mice to the swine erysipelas vaccine VR_2

Time of Retardillin administration \ Vaccine dose (number of living cells)	No. of vaccinated mice	No. of challenged mice	Mean protective efficiency ($\bar{x}\pm s$) $2 \cdot 10^3$ Protection (%)	P^*	$2 \cdot 10^4$ Protection (%)	P^*	$2 \cdot 10^5$ Protection (%)	P^*
Untreated	90	83	41.48± 2.56	—	78.52± 1.28	—	96.67±5.77	—
72 h before vaccination	90	83	41.11± 8.39	>0.01	82.13±10.53	>0.05	96.30±6.41	>0.05
24 h before vaccination	90	82	21.11± 9.56	<0.05	55.56±11.12	<0.05	92.13±6.85	>0.05
Simultaneously with vaccination	90	87	10.00± 0.00	<0.001	41.48±10.32	<0.01	71.67±2.89	<0.01
24 h after vaccination	90	86	16.67± 5.77	<0.01	41.85±12.88	<0.01	70.37±6.41	<0.01
72 h after vaccination	90	89	30.00±10.00	>0.05	83.33± 5.77	>0.05	92.96±6.12	>0.05

* Relative to vaccine control.

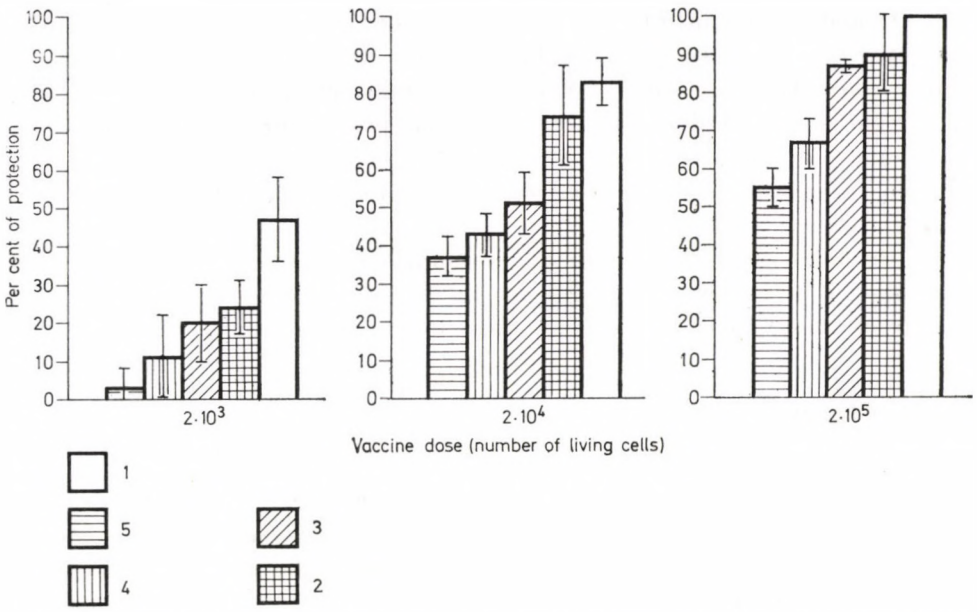
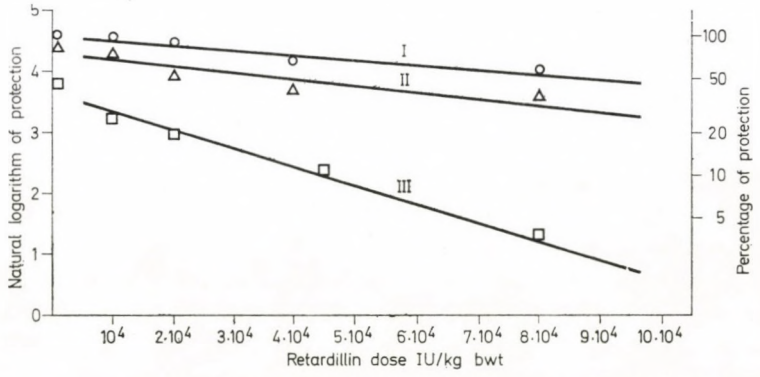


Fig. 2. Effect of different doses of Retardillin on the immune response of mice to the live swine erysipelas vaccine VR_2. 1. Vaccine alone; 2. Vaccine+10,000 IU/kg bwt Retardillin; 3. Vaccine+20,000 IU/kg bwt Retardillin; 4. Vaccine+40,000 IU/kg bwt Retardillin; 5. Vaccine+80,000 IU/kg bwt Retardillin



$$y_I = 97.93\, e^{-0.08 \cdot 10^{-4} x} \quad (\ln y_I = -0.08 \cdot 10^{-4} x + 4.58)$$

$$y_{II} = 74.02\, e^{-0.1 \cdot 10^{-4} x} \quad (\ln y_{II} = -0.1 \cdot 10^{-4} x + 4.30)$$

$$y_{III} = 38.94\, e^{-0.31 \cdot 10^{-4} x} \quad (\ln y_{III} = -0.31 \cdot 10^{-4} x + 3.66)$$

Fig. 3. Correlation between percentual mouse protecting efficiency and dose of Retardillin

Simultaneous antibiotic treatment unequivocally depressed the immune response to the vaccine, but the disadvantageous influence of Retardillin was the weaker the larger dose of vaccine had been administered.

The standard deviation of percentual protection was calculated, and inter-group differences relative to the vaccine control were analysed for significance. The responses differed from the control at $P < 0.05$—0.001 levels of significance.

It follows that under the given conditions of experiment the immune response of the vaccinees represented a function of both vaccine dose and antibiotic dose. This relationship can be expressed in an exponential form as

$$y = Be^{ax}$$

or in a logarithmic form as

$$\ln y = ax + b$$

where x is the actual Retardillin dose in terms of IU/kg bwt, y the immunity in terms of percentual survival, a the slope, b the intersection of the straight line and ordinate, and B the value of e^b. Under the given conditions, the following equations of regression were obtained for vaccine doses with $2 \cdot 10^5$, $2 \cdot 10^4$ and $2 \cdot 10^3$ bacterium count:

	Vaccine dose
$y_1 = 97.93\ e^{-0.08 \cdot 10^{-4}x}$	$2 \cdot 10^5$ germs
or	
$\ln y_1 = -0.08 \cdot 10^{-4}\ x$	
$y_2 = 74.02\ e^{-0.1 \cdot 10^{-4}x}$	
or	$2 \cdot 10^4$ germs
$\ln y_2 = -0.1 \cdot 10^{-4}\ x + 4.30$	
$y_3 = 38.94\ e^{-0.31 \cdot 10^{-4}x}$	
or	$2 \cdot 10^3$ germs
$\ln y_3 = -0.31 \cdot 10^{-4}\ x + 3.66$	

The protective value of the vaccine, expressed as the natural logarithm of the percentage of surviving vaccines, showed a negative linear regression with the antibiotic dose in terms of IU/kg bwt. The slopes of the graphically presented regression lines varied with the dose of the vaccine (Fig. 3).

Finally, the influence of the time of antibiotic administration on immune response was examined in mice treated with a constant dose of Retardillin (40,000 IU/kg bwt) 24 and 72 h before, simultaneously, or 24 and 72 h after immunization with the VR_2 vaccine. The degree of protection was expressed in survival per cent. The results are shown in Table V, which also includes standard deviations and information on the significance of differences from vaccine controls. It was found that the applied dose of Retardillin had no

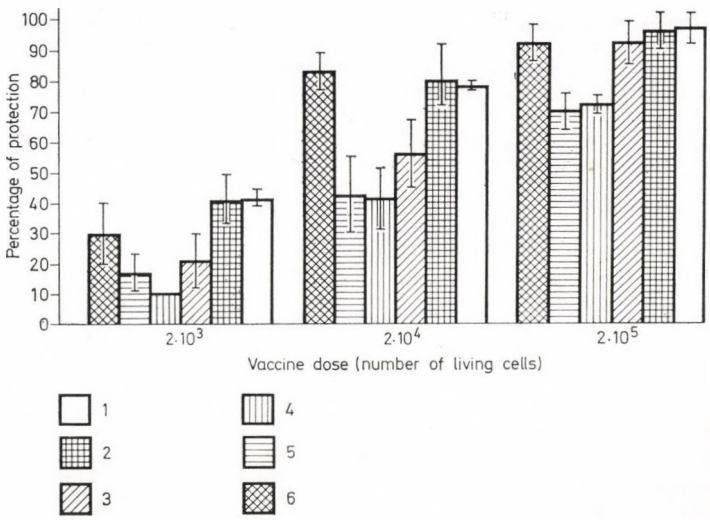


Fig. 4. Influence of the time interval between Retardillin treatment and vaccination on mouse immune response to the swine erysipelas vaccine VR_2. 1, Vaccine alone; 2, Retardillin treatment 72 h before vaccination; 3, Retardillin treatment 24 h before vaccination; 4, Retardillin treatment simultaneously with vaccination; 5, Retardillin treatment 24 h after vaccination; 6, Retardillin treatment 72 h after vaccination

significant influence on immune response when administered 72 h before or after vaccination, while it accounted for a considerable depression when given 24 h before simultaneously with, or 24 h after immunization. The strongest influence was observed if the antibiotic was given 24 h after immunization and the vaccine dose was small (Fig. 4).

The experimental observations have unequivocally supported the conclusion that treatment with the antibiotic Retardillin depressed the immune response of mice to vaccination with the low-virulence VR_2 strain of *E. rhusiopathiae*. The degree of depression depended on the size of the applied dose of vaccine and that of the antibiotic, as well as on the time of antibiotic administration relative to vaccination. Under the given conditions of experiment, the immune suppressive action of the antibiotic can be explained by the depression of the multiplication of the vaccine strain in the vaccinated organism.

The question may nevertheless arise whether the antibiotic administration also affects the immune system of the host *per se*, without any specific action on the pathogen. Experimental studies of this problem are the subject of the next report in this publication series.

Summary

A swine erysipelas vaccine prepared from the low-virulence *E. rhusiopathiae* vaccine strain VR_2 was examined in mouse protection tests for interaction with Retardillin, a preparation containing a penicillin derivative.

1. The vaccine strain and the virulent *E. rhusiopathiae* strain OK 22, used for challenge, showed *in vitro* identical sensitivity to penicillin. The *in vivo* mouse-protecting effect of Retardillin depended greatly on the number of germs introduced in the vaccine.

2. The immunity of VR_2-vaccinees to challenge with 1000 LD_{50} of the OK 22 strain also depended considerably on the dose of the vaccine: 10^2 VR_2 organisms living bacterial cells protected only 10.37%, while 10^5 cells protected 96.67% of the vaccinees against challenge. The protective value of the vaccine, expressed in terms of survival per cent, showed a positive linear relationship with the logarithm of the vaccine dose in terms of bacterium. Further increase of the immunizing dose is prevented by the residual virulence of the strain VR_2.

3. The response of mice treated simultaneously, but at different sites, with VR_2 vaccine plus rising doses of Retardillin, to challenge with 1000 LD_{50} of the virulent strain two weeks later, depended partly on the dose of the vaccine, partly on that of the antibiotic. Protection against challenge infection showed a negative exponential relationship with the applied dose of Retardillin in terms of IU/kg bwt.

4. The immune response of VR_2+antibiotic treated mice also depended on the length of the time interval between vaccination and antibiotic administration. A constant dose of Retardillin (40,000 IU/kg bwt) depressed immune response to vaccination to a greater degree when applied 24 h before VR_2 than 24 h after it, especially if the vaccine dose was high. Antibiotic treatment applied 72 h before or after vaccination had no notable influence on immunogenicity. Depression of immune response by the antibiotic greatly depended on the dose of the vaccine.

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EFFECTS OF ANTIBIOTICS ON THE IMMUNE SYSTEM OF ANIMALS

II. MOUSE EXPERIMENTS WITH AN INACTIVATED ADSORBED VACCINE AGAINST SWINE ERYSIPELAS

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(Received October 31, 1978)

It was reported in the previous publication of this series that simultaneous treatment with the penicillin derivative Retardillin depressed the immune response of mice to vaccination with the low-virulence swine erysipelas strain VR_2 (TRAN DIHN TU, 1979).

In further studies, I investigated whether the immune-depressant effect was due to antibiotic action on the live bacteria introduced by vaccination in particular, or to a nonspecific immunodepressive influence of the antibiotic in general.

The influence of antibiotics on immune responsiveness has been extensively studied by both medical and veterinary investigators, but contradictory observations have been reported in both fields. Among others, the effect of antibiotics on immunization with erythrocytes has been differently interpreted. Certain authors found that simultaneous administration of erythrocytes and streptomycin enhanced the former's immunogenicity, *i.e.* the serum haemolysin level rose over the control (FARHI and LAMESANS, 1956). Other investigators (HARMON et al., 1973) have stated the opposite. Treatment of erythrocyte-immunized mice with penicillin or streptomycin one hour after immunization depressed not only the haemagglutinin titre, but also the production of the antibody-producing cells (NIKOLAYEV et al., 1967; NIKOLAYEV and NAZARMUKHAMEDOVA, 1974).

Rabbits simultaneously treated with ^{131}I-labeled bovine gamma globulin as antigen and different antibiotics (penicillin, streptomycin, terramycin) showed in most cases a diminished immune responsiveness in comparison to controls, as judged by the rate of disappearance of the labeled antigen from the blood (STEVENS, 1953).

Many authors used inactivated vaccines (by formalin, heat, *etc.)* prepared from different salmonella strains in model experiments, in which oral treatment of the vaccinees with tetracycline on a single occasion or on several consecutive days usually depressed both the humoral (agglutination titre) and cellular immune response (lymphocyte activity) to vaccination (CHUMACHENKO and SOKOLOVA, 1962; PROCHÁZKA et al., 1968; CHERNIGOV, 1972; LAKHOTIA and STEPHENS, 1972). One author (AVERIANOVA, 1957) reported that antibiotics applied after administration of inactivated salmonella antigen had no demonstrable influence on the serum agglutinin titre of dogs, and although pigs responded to subcutaneous administration by decrease of the antibacterial agglutinin titre, they simultaneously showed an increase in serum haemagglutinin. Oral administration of both antigen and antibiotic resulted only in a delay of antibody synthesis in comparison to controls (SCHOLLENBERGER, 1971). HARMON et al. (1973) reported that oral treatment of pigs with zinc bacitracin, cycloserine or neomycin simultaneously with the parenteral application of salmonella antigen even accounted for a significant increase in the antibody response over the controls.

Inactivated swine erysipelas vaccines were also studied for *in vivo* interaction with antibiotics. Formerly, authors generally agreed that pigs given antibiotic simultaneously with

the adsorbed vaccine or on long term by the oral route were not inferior to the controls in respect of immune response to challenge three weeks later (ALLEN and COOPER, 1956; WILLINGER and STÖCKL, 1961, 1962; WILLINGER et al., 1962). Recent findings have, however, increasingly indicated that oral treatment with antibiotic for several consecutive days during the period of vaccination had an adverse influence on immune response, *viz.* retarded synthesis and low titre of serum agglutinins, and that the degree of depression depended on the dose and duration of antibiotic treatment (LYASHENKO, 1966; FORTUSHNIY et al., 1973).

The partly contradictory findings prompted us to investigate the problem in detail.

We performed model experiments in mice immunized with an adsorbed swine erysipelas vaccine to establish the influence on their immune response of treatment with the antibiotic preparation Retardillin given in different doses and at different times.

Materials and methods

Vaccine: A commercial adsorbed swine erysipelas vaccine preparation (PHYLAXIA Veterinary Biologicals and Feedstuffs Co. Ltd., Budapest).

Antibiotic: Retardillin® (benzyl-penicillin-procaine; BIOGAL, Debrecen). Saline was used as diluent.

Experimental animals: Albino mice of both sexes, 20 ± 2 g in weight.

Vaccine immunogenicity was tested with the procedure used for efficacy testing (GELENCZEI et al., 1953).

The vaccine was diluted in 1% aluminium hydroxide gel, at fourfold serial steps (Range: 4°—4^{-3}). Five mice were immunized subcutaneously with 0.2 ml doses of each dilution. Fourteen days later the vaccinees were challenged with 1000 LD_{50} of the virulent *E. rhusiopathiae* strain OK 22 (see previous publication of the series), also by the subcutaneous route. The animals were then observed for 10 days, during which losses were recorded. The presence of *E. rhusiopathiae* in carcases was demonstrated by re-isolation from the heart blood. The vaccines were treated either with rising doses of the antibiotic (range: 10,000—80,000 IU/kg bwt) simultaneously to vaccination, or with a constant intramuscular dose (80,000 IU/kg bwt), but at different times relative to the latter.

An untreated control group and one vaccinee group not treated with antibiotic (vaccine control) was set up with each test. The intensity of infection was checked by simultaneous titration. Each experiment was performed in three replica series.

Evaluation of the immune responses and mathematical-statistical analysis of the findings

The 50% mouse protective dose of the vaccine was calculated from the losses by the method of REED and MUENCH (1938), and it was expressed in

terms of immunizing unit(s) (IU). The value of the vaccine was classified as one IU if 1 ml of it protected 50% of the vaccinated mice against infection with *E. rhusiopathiae* challenge.

Mean value and standard error were calculated for each experimental group, and inter-group differences were analysed for significance by Student's t test. Correlation coefficients were determined.

Results

In the first experimental series different doses of Retardillin were administered simultaneously with the adsorbed swine erysipelas vaccine. The effects of this treatment on immune response are shown in Table I and Figs 1 and 2.

Table I

Effect of different doses of Retardillin on the immune response of mice to vaccination with an inactivated adsorbed swine erysipelas vaccine

Dose of Retardillin (IU/kg bwt)	No. of mice		Immunity of mice in immune units ID_{50}	Significance of difference from vaccine control P
	vaccinated	challenged		
Vaccine alone	60	60	97.73±15.69	—
Vaccine+10,000 IU/kg	60	57	98.64±25.47	0.05
Vaccine+20,000 IU/kg	60	59	68.92±20.79	0.05
Vaccine+40,000 IU/kg	60	58	40.92±19.30	0.05
Vaccine+80,000 IU/kg	60	60	25.62±12.55	0.01

Taking the protective value found in the vaccine control group as 100, the relative percentages of protection were 29.5, 58.2 and 73.9% among the mice treated with Retardillin at 20,000, 40,000 and 80,000 IU/kg bwt level. In 10,000 IU/kg bwt dose, Retardillin had no influence on the immune response. The difference between the immunodepressant effects of the different antibiotic doses was statistically significant. Under the given conditions of experiment the degree of protection by the vaccine against later challenge depended on the dose level of the simultaneously administered Retardillin. This correlation can be expressed in an exponential form as

$$y = Be^{ax}$$

or in a logarithmic form as

$$\ln y = ax + b,$$

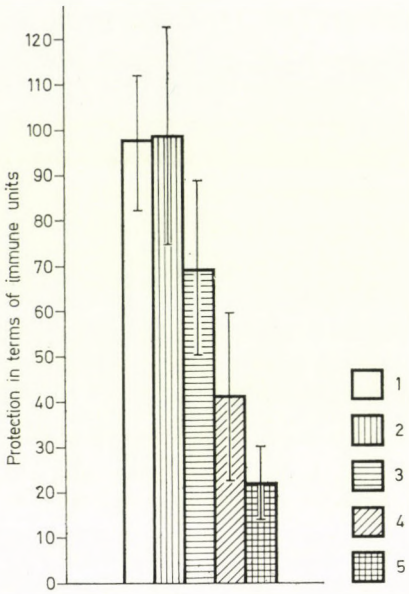

Fig. 1. Effect of different doses of Retardillin on the immune response of mice to an inactivated adsorbed swine erysipelas vaccine. 1, Vaccine alone; 2, Vaccine+10,000 IU/kg; 3, Vaccine+20,000 IU/kg; 4, Vaccine+40,000 IU/kg; 5, Vaccine+ 80,000 IU/kg

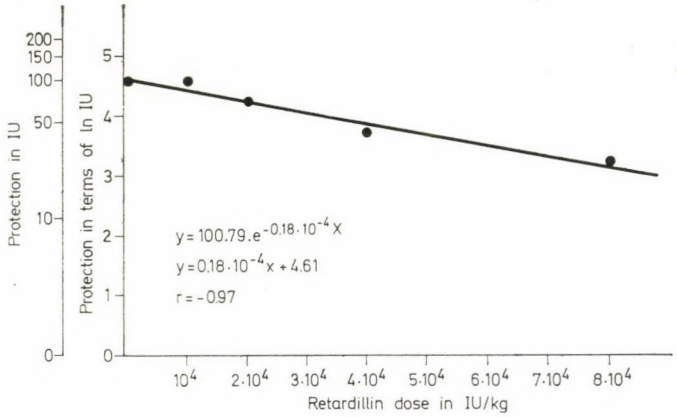

Fig. 2. Correlation between the protection of mice against swine erysipelas and the applied Retardillin dose

where x represents Retardillin dose in terms of IU/kg bwt, y the degree of protection of the mice in terms of ID_{50}, a the tangent slope, b the intersection of the straight line and ordinate, and B the value of e^b. Substituting the experimental data, we have the regression equation

$$y = 100.79\, e^{-0.18 \cdot 10^{-4}x}$$

or

$$\ln y = -0.18 \cdot 10^{-4} x + 4.61$$

The correlation coefficient,

$$r = -0.97.$$

It follows that the protective value of the absorbed vaccine, in terms of IU units, showed a negative exponential correlation with the dose of Retardillin in terms of IU/kg bwt.

Subsequently, the effect of the time of antibiotic administration on immune response to vaccination was examined in mouse groups given an identical dose of Retardillin (80,000 IU/kg bwt) simultaneously with the vaccination, or at 24 and 72 h before or after it. The results obtained in three experimental series are shown in Table II and Fig. 3.

Table II

Effect of the time of Retardillin administration relative to vaccination on the immune response of mice to an inactivated adsorbed swine erysipelas vaccine

Time of Retardillin administration (80,000 IU/kg bwt)	No. of mice		Immunity of mice in immune units ID_{50}	Significance of difference from vaccine control P
	vaccinated	challenged		
Vaccine alone	60	60	54.04± 4.33	—
72 h before vaccination	60	59	59.58±20.15	0.05
24 h before vaccination	60	60	38.10± 7.21	0.05
Simultaneously with vaccination	60	59	16.71± 3.27	0.001
24 h after vaccination	60	60	22.95± 5.11	0.01
72 h after vaccination	60	58	36.03± 8.56	0.05

The antibiotic treatment had no notable influence on immunity, if it was applied 72 h before the vaccination, but it accounted for a minor to major depression if administered later. Strictly speaking, with the immunity of the mice treated with vaccine only taken as 100, the percentage of protected animals fell to 29.5, 69.1, 57.5 and 33.4% respectively, in the mice groups given antibiotic 24 h before vaccination, simultaneously with it, and at 24 and 72 h after it, *i.e.* the depressive effect of Retardillin was greatest on simultaneous administration, scarcely less at 24 h after vaccination, and least pronounced, but still marked, at 24 h before and 72 h after the vaccine.

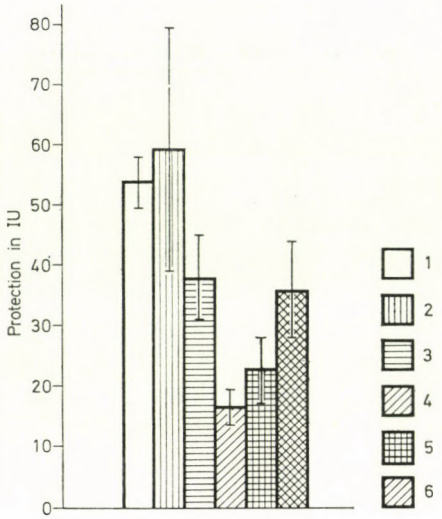

Fig. 3. Effect of the time of Retardillin administration relative to vaccination on the immune response of mice to an inactivated adsorbed swine erysipelas vaccine. 1, Vaccine alone; 2, 72 h before vaccination; 3, 24 h before vaccination; 4, simultaneously with vaccination; 5, 24 h after vaccination; 6, 72 h after vaccination

Discussion

The experimental observations have unequivocally shown that antibiotic treatment has an immunosuppressive effect on the living organism. Administration of antibiotic in the period of vaccination depressed the immunogenicity not only of the live, but also of the inactivated swine erysipelas vaccine. The degree of immunosuppression depended on the dose of the antibiotic, and on its time relative to vaccination as well. The immunosuppressive effect was of short duration and reversible, for it lasted only until elimination of the antibiotic from the vaccinated organism. Thus administration of the antibiotic 72 h before vaccine treatment did not affect immunogenicity, but 72 h after it developed a measurable adverse action on the still operative immune mechanism, *i.e.* it temporarily inhibited the blast transformation of small lymphocytes (GERGELY and OTT, 1974).

The conflicting findings reported in the literature on antibiotic-vaccine interaction can be attributed to differences between the types of antigen and antibiotic tested, and to species differences between the experimental animals as well. Gross differences in the conditions of experiment can thus account for contradictory observations.

Model experiments in mice cannot satisfy the practical requirements. I therefore set up pig experiments to obtain reliable information on antibiotic effect on immunization against swine erysipelas. The pig experiments will be the subject of the forthcoming report of this series.

Summary

The effect of simultaneous antibiotic administration on the immunogenicity of inactivated adsorbed swine erysipelas vaccine was examined in model experiments in mice.

Mice treated with the adsorbed vaccine and simultaneously with rising doses (20,000, 40,000 and 80,000 IU/kg bwt) of the antibiotic Retardillin showed a dose-dependent decrease of percentual protection (by 29.5%, 58.2%, and 73.2%, respectively) against challenge with 1000 LD_{50} virulent *E. rhusiopathiae* two weeks later, relative to those treated with the vaccine alone (100%).

It follows that simultaneous antibiotic treatment depressed the immunogenic effect not only of live, but also of inactivated bacterial antigen. The immuno-suppressive action tended to increase as the antibiotic dose was risen. The mouse protective value of the vaccine showed a negative exponential correlation with the applied dose of Retardillin.

The immuno-suppressive action of the antibiotic also depended on the time of its administration relative to vaccination. Treatment with the same large dose of Retardillin at different times showed that immuno-suppression was greatest on simultaneous administration of the vaccine and antibiotic. This accounted on the average for a 61.9% decrease in the proportion of protected mice relative to those given vaccine alone. Administration of antibiotic 24 h before and after vaccination reduced the percentage of protection by 29.5 and 57.5%, respectively. When given 72 h after vaccination, the antibiotic still reduced immunogenicity by 33.4%, but it had no influence when administered 72 h before the vaccine.

It follows that the antibiotic treatment interfered with the immune mechanism of the organism reversibly, but long enough to suppress the immunogenic action of the inactivated adsorbed swine erysipelas vaccine.

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EFFECT OF ANTIBIOTICS ON THE IMMUNE SYSTEM OF ANIMALS

III. EXPERIMENTS IN PIGS IMMUNIZED WITH LIVE AND INACTIVATED SWINE ERYSIPELAS VACCINES

By

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I have shown in model experiments, that treatment of mice with the antibiotic Retardillin® (benzyl-penicillin-procaine) depressed immune response to both live (VR_2) and inactivated swine erysipelas vaccine.

Further experiments were performed in pigs treated with either vaccine plus Retardillin to obtain information on the practical aspects of antibiotic influence on pig immune response to vaccination against swine erysipelas.

The problem has been studied by several veterinary investigators, but different conditions of experiment or field study have led to contradictory conclusions. Certain authors established that lasting feed supplementation with antibiotic did not interfere with immune response to live swine erysipelas vaccines (OSE et al., 1963; KOVALYEV, 1964), while many others (WILLINGER et al., 1961; STÖCKL and WILLINGER, 1962; KOVALENKO and TATARINCHEV, 1962; LYASHENKO, 1965, 1966) stated the opposite. It was concluded that dietary antibiotic supplies should be preferably omitted during immunization against swine erysipelas with a live vaccine. Authors experimenting with therapeutic doses of different antibiotics in pigs treated with live vaccine or infected experimentally with a virulent strain arrived at the same conclusion (KOVALYEV, 1966; FORTUSHNIY et al., 1966, 1973; AZECHI et al., 1972).

The influence of the time interval between antibiotic treatment and vaccination has been investigated by numerous authors; for details and references see the first publication of this series (TRAN DINH TU, 1979a).

Some authors found no indication of immunosuppression by the antibiotic if an inactivated adsorbed swine erysipelas vaccine was used for immunization, but Soviet investigators reported a markedly poor immune response in pigs so vaccinated simultaneously with oral tetracyclin treatment (for details see Part II of this series).

Experimental evidence of the direct immunosuppressive action of antibiotics has emerged from JOCHEV's (1962) observation that penicillin treatment of pigs was followed by a marked decrease in phagocytic activity, especially during the first hours.

The equivocal reports prompted closer investigations into the problem.

In the present study, pigs immunized against swine erysipelas either with the VR_2 strain of reduced virulence, or with an inactivated adsorbed preparation, were treated with Retardillin® either simultaneously with the vaccination at different dose levels, or with an equal dose at different times relative to vaccination, to assess the influence of the antibiotic on the immune response.

Materials and methods

Erysipelothrix rhusiopathiae *strains*

Vaccine strain: The type 2a strain VR_2, of reduced virulence, available in freeze-dried conditions, was used.

Strain used in agglutination test: a type 1a laboratory strain designated as "Marienfelde".

Virulent strains: type 1a strains isolated from pigs died of septicaemic erysipelas.

Inactivated adsorbed swine erysipelas vaccine: a commercially available preparation produced by the PHYLAXIA Veterinary Biologicals and Feedstuffs Co. Ltd., Budapest.

Antibiotic: Retardillin^R (benzyl-penicillin-procaine), BIOGAL, Debrecen.

Experimental animals

Susceptible growing pigs of the Hungarian White Bacon breed, 27.3 ± 4.6 kg in weight, not previously immunized and serologically negative, were used in groups of 3.

Immunization

The VR_2 vaccin was applied subcutaneously in two experiments, at the individual dose levels of $2 \cdot 10^9$ (Tables I and II) and $0.5 \cdot 10^9$ cells (Tables V and VI), respectively.

Among a total of 60 pigs treated with the VR_2 vaccine, 11 developed within 3—7 days a mild febrile urticaria, which subsided spontaneously after 2—3 days. The mild vaccination disease had no bearing either on immunity to challenge, or on serum antibody levels.

The inactivated adsorbed vaccine was administered subcutaneously, in the recommended dose, *i.e.* 5 ml. This vaccine did not give rise to vaccination disease in any case.

In the first experiment the pigs vaccinated with the VR_2 strain were simultaneously given 10,000, 40,000 or 160,000 IU/kg bwt Retardillin by the intramuscular route. In the second experiment each animal treated with VR_2 received equally 40,000 IU/kg bwt, and each treated with adsorbed vaccine 160,000 IU/kg bwt of Retardillin intramuscularly, at different times relative to vaccination.

Blood samples were taken from the pigs before vaccination and once every week after it (on 4 occasions) for serological testing. The samples were stored at —20 °C until use.

On a total 144 pigs were used.

Challenge

Immunity was challenged 21 days after vaccination, by rubbing into the depilated scarified skin of vaccinees a 24 h broth culture of virulent *E. rhusiopathiae* as proposed by FORTNER and DINTER (1944). The animals were observed for 7 subsequent days, during which body temperature, cutaneous reactions and general symptoms were recorded daily. The cutaneous reaction was evaluated in the four-cross system (+, ++, +++, ++++). The pig exhibiting $\geq$1 °C rise in body temperature over normal was regarded as febrile.

Serological test

The serological assay of immunity to swine erysipelas was based on WELLMAN's culture agglutination test (1955) as modified by KUCSERA (1960). To the horse meat broth used as culture medium sodium azide and crystal violet were added at the final concentration of 1 : 2000 and 1 : 400,000, respectively, to prevent the growth of contaminating bacteria. The serum samples were diluted directly in the culture medium, at twofold serial steps within the range of 1 : 2 to 1 : 1024. As antigen one drop of a 24 h broth culture of the strain "Marienfelde" was added to each serum dilution. The reaction was read after 24 h incubation at 37 °C. The positive reaction was scored with one to four crosses, depending on the intensity of agglutination. The highest dilution exhibiting an at least two-cross reaction was regarded as the end titre. Each blood sample was tested in two replicas, and the mean value was calculated.

Evaluation of immune response and statistical analysis

The immune state of the vaccinees was classified into three categories (KUCSERA, 1962) on the basis of their response to challenge:

immune — pigs without clinical symptoms

partially immune — pigs exhibiting a slight cutaneous reaction, and

susceptible — pigs with a severe cutaneous reaction and fever.

For graphical presentation the base-2 logarithms of the reciprocal serological titres were plotted against time. From these were calculated the mean antibody titres and the standard error. Changes in antibody titres were analysed by Student's t test. The attached Tables show the geometric mean values of the titres and the 95% fiducial limits.

Results

The results of simultaneous administration of VR_2 vaccine with different doses of Retardillin are shown in Tables I and II and Fig. 1.

Table I

Influence of simultaneous treatment with live VR_2 swine erysipelas vaccine and different doses of Retardillin on pig immune response

Retardillin dose (IU/kg bwt)	No. of pigs	Post-challenge symptoms							Response of vaccines to challenge		
		Cutaneous reaction					Fever	Inappetence	Immune	Partially immune	Susceptible
		Negative	+	++	+++	++++					
Vaccine alone	6	5	1	0	0	0	0	0	5	1	0
Vaccine+10,000 IU/kg bwt	6	4	0	1	0	1	1	0	4	1	1
Vaccine+40,000 IU/kg bwt	6	4	0	0	1	1	2	0	4	0	2
Vaccine+160,000 IU/kg bwt	5*	0	1	0	1	3	4	0	0	1	4
Unvaccinated control	6	0	0	0	0	0	6	6	2	0	0

* One pig died of intercurrent disease before challenge.

Table II

Agglutinin titre changes in the sera of pigs simultaneously treated with live VR_2 swine erysipelas vaccine and different doses of Retardillin

Dose of Retardillin (IU/kg bwt) / Time of blood sampling (week)	No. of pigs	Geometric mean of reciprocal antibody titres (95% fiducial limits)				
		Before vaccination	One week after vaccination	Two weeks after vaccination	Three weeks after vaccination	One week after challenge
Vaccine alone	6	9.8 (5.8—16.4)	171.3 (122.0—241.2)	115.4 (65.4—203.5)	48.9 (23.1—103.4)	192.7 (141.1—263.5)
Vaccine+10,000 IU/kg bwt	6	8.6 (3.4—21.9)	85.7 (29.1—253.2)	79.4 (23.1—273.3)	44.3 (14.0—139.9)	145.1 (52.4—401.6)
Vaccine+40,000 IU/kg bwt	6	10.8 (7.5—15.5)	69.6* (33.4—145.1)	69.6* (28.1—172.7)	45.9 (17.4—120.8)	148.1 (77.7—282.9)
Vaccine+160,000 IU/kg bwt	6	10.0 (5.4—18.4)	24.1** (11.8— 49.1)	16.5** (8.2— 33.1)	12.4* (6.1— 25.3)	188.8 (111.5—318.7)
Unvaccinated control	6	9.2 (6.3—13.3)	9.2*** (5.1— 16.6)	7.8*** (4.2— 14.3)	8.2*** (4.9— 13.7)	73.1* (34.5—154.5)

* $P<0.05$.
** $P<0.01$ Significance of difference from titres of pigs treated with vaccine alone.
*** $P<0.001$.

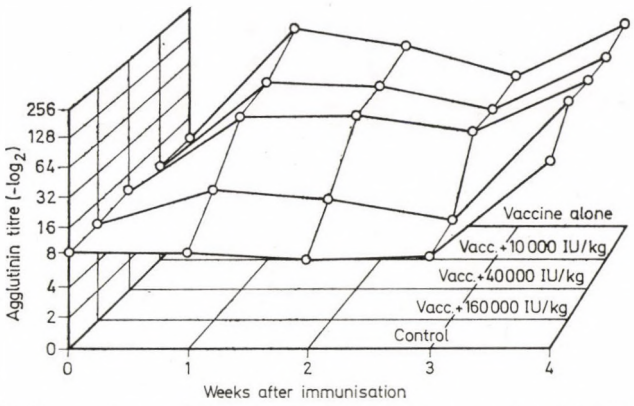

Fig. 1. **Effect of different doses of Retardillin given simultaneously with VR_2 vaccine on the serum titres in the culture agglutination test**

The influence of Retardillin on the immune response to $2 \cdot 10^9$ VR_2 organisms tended to increase as the antibiotic dose was risen.

Treatment with a single dose of 10,000 IU/kg bwt Retardillin did not interfere with pig immune response, but 40,000 IU/kg bwt depressed it markedly, and the vaccinees given 160,000 IU/kg bwt of the antibiotic responded to challenge with virulent bacteria 21 days later exactly as the unvaccinated controls.

The serological tests disclosed that pigs given vaccine but no antibiotic showed a rapid rise of the serum agglutinin titre, which reached its peak in a week and gradually decreased thereafter until challenge.

The agglutinin titre curves for vaccines given 10,000 or 40,000 IU/kg bwt Retardillin followed the same trend, but at lower levels depending on the dose of the antibiotic. The titre curve for the group treated with 160,000 IU/kg bwt Retardillin scarcely differed from that obtained for unvaccinated controls (Fig. 1). The agglutinin titres of pigs given vaccine alone and vaccine+40,000 IU/kg bwt Retardillin differed significantly ($P<0.05$) only during the first postvaccination week, while at the highest level of treatment the difference from the vaccine control remained significant ($P<0.01$—0.05) as long as three weeks.

The results of the second experiment, in which administration of the adsorbed vaccine was combined with simultaneous Retardillin treatment, are shown in Tables III and IV, and Fig. 2.

Protection tests disclosed that vaccinees given 10,000 or 40,000 IU/kg bwt Retardillin responded to challenge similarly to pigs treated with vaccine alone, *i.e.* by a slight cutaneous reaction. However, among the vaccines given 160,000 IU/kg bwt of the antibiotic, only 50% were immune to challenge, the remaining developed a severe cutaneous reaction; one animal even had a febrile course.

Table III

Influence of simultaneous treatment with inactivated adsorbed swine erysipelas vaccine and different doses of Retardillin on pig immune response

Retardillin dose (IU/kg bwt)	No. of pigs	Post-challenge symptoms							Response of vaccinees to challenge		
		Cutaneous reaction					Fever	Inappetence	Immune	Partially immune	Susceptible
		Negative	+	++	+++	++++					
Vaccine alone	6	4	1	1	0	0	0	0	4	2	0
Vaccine+10,000 IU/kg bwt	6	4	1	0	1	0	0	0	4	2	0
Vaccine+40,000 IU/kg bwt	6	4	0	1	1	0	0	0	4	2	0
Vaccine+160,000 IU/kg bwt	6	3	0	0	2	1	1	0	3	2	1
Unvaccinated control	6	0	0	0	0	6	6	6	0	0	6

Table IV

Agglutinin titre changes in the sera of pigs simultaneously treated with inactivated adsorbed swine erysipelas vaccine and different doses of Retardillin

Time of blood sampling (week) / Dose of Retardillin (IU/kg bwt)	No. of pigs	Geometric means of reciprocal antibody titres (95% fiducial limits)				
		Before vaccination	One week after vaccination	Two weeks after vaccination	Three weeks after vaccination	One week after challenge
Vaccine alone	6	5.4 (3.1—9.6)	11.0 (8.3—14.6)	12.4 (9.5—16.2)	17.2 (14.4—20.4)	73.0 (50.2—105.8)
Vaccine+10,000 IU/kg bwt	6	4.3 (3.6—5.1)	9.8 (5.8—16.4)	11.0 (5.8—21.0)	18.4 (8.4—38.9)	55.4 (27.5—111.3)
Vaccine+40,000 IU/kg bwt	6	4.3 (3.6—5.1)	5.6** (3.7— 9.0)	8.8 (6.5—11.8)	13.9 (9.4—20.3)	54.2 (40.2—73.0)
Vaccine + 160,000 IU/kg bwt	6	7.3 (6.2—8.5)	8.6 (6.1—12.1)	8.8 (5.8—13.2)	11.6* (8.3—16.0)	51.3 (34.3—76.5)
Unvaccinated control	6	5.1 (3.5—6.6)	5.1*** (3.5— 6.6)	4.5*** (3.3— 6.1)	4.5*** (3.3— 6.1)	48.5 (30.5—77.3)

* $P<0.005$.
** $P<0.01$ Significance of difference from titres of pigs treated with vaccine alone.
*** $P<0.01$.

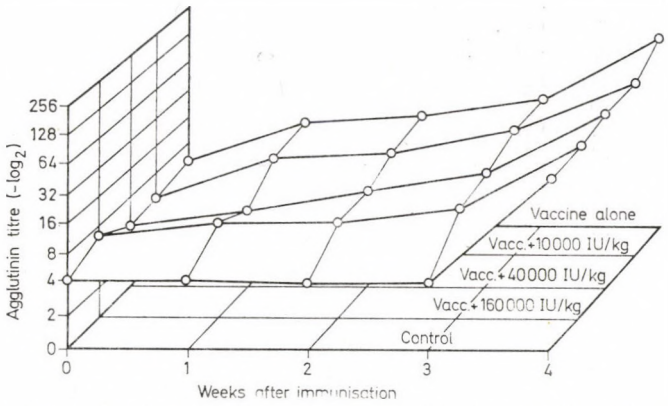

Fig. 2. Effect of different doses of Retardillin given simultaneously with swine erysipelas adsorbate vaccine on the serum titres in the culture agglutination test

The serological tests showed that the appearance of circulating antibodies followed considerably later on immunization with the adsorbed than with the live vaccine. The pigs treated with adsorbed vaccine had consequently much lower serum agglutinin titres during the first weeks than the VR_2-vaccines. The titre peak was reached during the third postvaccination week in all groups.

The titre differences proved to be statistically significant (Table IV). The inter-group differences are still more obvious if the prevaccination agglutinin titre was uniformly regarded as 100% in each group. By this approach it became readily obvious that simultaneous administration of 40,000 and, especially, of 160,000 IU/kg bwt Retardillin depressed the immune response to the adsorbed vaccine. The titre decreased by about 50% at the highest level of antibiotic treatment.

The influence of different times of Retardillin treatment relative to immunization with the live or the adsorbed vaccine was also examined in pigs. An identical dose of antibiotic was therefore administered 72 and 24 h before and after vaccination, as well as simultaneously with it.

The response of VR_2-vaccines to administration of the same dose of antibiotic at different times is shown in Tables V and VI, and in Fig. 3.

Treatment with 40,000 IU/kg bwt Retardillin 72 h before live vaccination had no notable influence on the immune response, but both pre- and postvaccination treatment at 24 h depressed it considerably, and the 72 h postvaccination dose still had a measurable immunosuppressive effect. Maximum suppression was found after the 24 h postvaccination treatment (Table V).

The serum agglutinin titres of the vaccinees showed a slower rise at the lower ($0.5 \cdot 10^9$) than at the higher ($2 \cdot 10^9$) dose level of VR_2 (Table VI). The titre reached its peak during the second postvaccination week, then tended

Table V

Effect of the time of Retardillin treatment relative to immunization with live VR_2 vaccine on pig response to challenge with virulent *E. rhusiopathiae*

Time of Retardillin administration (Dose: 40,000 IU/kg bwt)	No. of pigs	Post-challenge symptoms							Response of Vaccinees to challenge		
		Cutaneous reaction					Fever	Inappetence	Immune	Partially immune	Susceptible
		Negative	+	++	+++	++++					
Vaccine alone	6	6	0	0	0	0	0	0	6	0	0
72 h before vaccination	6	4	1	1	0	0	1	0	4	1	1
24 h before vaccination	6	2	1	0	3	0	3	2	2	1	3
Simultaneously with vaccination	6	2	0	2	0	2	2	1	2	2	2
24 h after vaccination	6	0	0	0	3	3	5	4	0	1	5
72 h after vaccination	6	2	0	1	3	0	1	1	2	3	1
Unvaccinated control	6	0	0	0	0	6	6	6	0	0	6

Table VI

Effect of different times of antibiotic administration relative to vaccination on the serum agglutinin titres of pigs immunized with live VR_2 swine erysipelas vaccine

Time of blood sampling (weeks) / Dose of Retardillin (I^{U}/kg bwt)	No. of pigs	Geometric means of reciprocal antibody titres (95% fiducial limits)				
		Before vaccination	One week after vaccination	Two weeks after vaccination	Three weeks after vaccination	One week after challenge
Vaccine alone	6	4.6 (2.8—7.6)	32.6 (11.3—95.2)	50.8 (28.1— 92.5)	29.6 (15.6—56.7)	78.3 (36.3—169.2)
72 h before vaccination	6	4.3 (2.6—7.0)	25.9 (13.6—49.3)	48.4 (19.3—122.2)	25.4 (10.6—61.4)	86.3 (48.2—154.3)
24 h before vaccination	6	5.1 (3.8—7.0)	12.9 (6.0—27.9)	28.5 (7.5—107.7)	20.5 (5.2—80.6)	86.3 (32.0—232.0)
Simultaneously with vaccination	6	4.8 (2.8—8.3)	13.6 (7.1—25.9)	48.4 (10.8—218.7)	27.7 (9.1—84.2)	98.6 (57.7—167.3)
24 h after vaccination	6	7.1 (5.4—9.6)	10.8* (6.0—19.3)	12.1** (6.2— 23.9)	9.0** (6.7—12.1)	96.8 (58.9—160.3)
72 h after vaccination	6	5.4 (3.8—7.8)	18.3 (11.4—29.3)	43.1 (16.0—116.0)	24.7 (13.4—45.3)	76.8 (35.3—167.0)
Unvaccinated control	6	5.7 (3.8—8.4)	4.8** (2.8— 8.3)	4.0*** (2.5— 6.3)	4.5*** (3.3— 6.1)	48.4 (24.6— 95.4)

* $P<0.05$.
** $P<0.01$ (Significance of difference from titres of pigs treated with vaccine alone).
*** $P<0.001$.

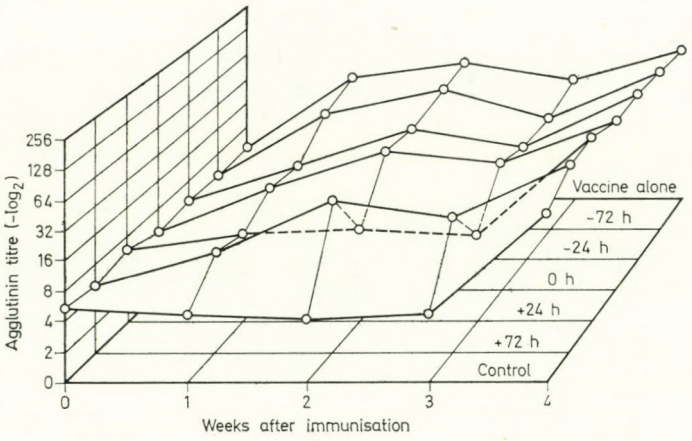

Fig. 3. Effect of Retardillin given simultaneously at different dates compared to vaccination with VR_2 vaccine on the serum titres in the culture agglutination test

to decrease gradually. The agglutinin titres of pigs given antibiotic 72 h before vaccination did not differ from those of animals treated with vaccine alone. Administration of the antibiotic 24 h before, 72 h after, and simultaneously with vaccination accounted for retardation of the humoral immune response during the first postvaccination week, but after another week the serum antibody levels approximated those of the pigs given vaccine alone.

The inter-group titre differences were statistically not significant. Treatment with Retardillin 24 h after vaccination depressed the antibody synthesis completely. The mean titre of pigs so treated differed significantly from that of vaccines not given antibiotic.

The agglutinin titres of pigs treated with the adsorbed vaccine and with Retardillin 160,000 IU/kg bwt at different times relative to immunization are shown in Tables VII and VIII and Fig. 4.

Pigs treated with the antibiotic 72 h before the vaccine responded to challenge similarly to those given vaccine alone (Table VII). Administration of the antibiotic 24 h before, 72 h after, or simultaneously with vaccination accounted for a slight depression of immune responsiveness, but Retardillin treatment 24 h after the vaccination had a marked immunosuppressive effect.

Serological examinations (Table VIII) showed that antibiotic treatment 72 and 24 h before, and 72 h after vaccination scarcely modified the agglutinin titre curve relative to the vaccine control, while simultaneous and 24 h postvaccination treatments accounted for marked alterations thereof.

Titration of the serum samples withdrawn one week after challenge strongly suggested that neither the size of the antibiotic dose, nor the time of antibiotic administration had a notable influence on secondary response to challenge.

Table VII

Effect of the time of Retardillin treatment relative to immunization with adsorbed swine erysipelas vaccine on pig immune response to challenge with virulent *E. rhusiopathiae*

Time of Retardillin administration (Dose: 160,000 IU/kg bwt)	No. of pigs	Post-challenge symptoms							Response of vaccinees to challenge		
		Cutaneous reaction					Fever	Inappetence	Immune	Partially immune	Susceptible
		Negative	+	++	+++	++++					
Vaccine alone	6	3	2	1	0	0	0	0	3	3	0
72 h before vaccination	6	2	2	1	1	0	0	0	2	4	0
24 h before vaccination	6	2	2	1	1	0	1	0	2	3	1
Simultaneously with vaccination	6	2	1	2	1	0	1	0	2	3	1
24 h after vaccination	6	1	3	0	2	0	2	0	1	3	2
72 h after vaccination	6	2	2	0	2	0	1	0	2	3	1
Unvaccinated control	6	0	1	0	0	5	5	5	0	1	5

Table VIII

Effect of different times of antibiotic treatment relative to vaccination on the serum agglutinin titres of pigs immunized with inactivated adsorbed swine erysipelas vaccine

Time of Retardillin treatment (Dose: 160,000 IU/kg bwt) \ Time of blood sampling (week)	No. of pigs	Geometric means of reciprocal antibody titres (95% fiducial limits)				
		Before vaccanation	One week after vaccination	Two weeks after vaccination	Three weeks after vaccination	One week after challenge
Vaccine alone	6	6.1 (4.3— 8.7)	12.9 (9.6—17.3)	19.6 (10.8—35.5)	23.1 (14.8—36.0)	122.0 (55.7—248.8)
72 h before vaccination	6	6.8 (4.3—10.7)	14.5 (9.8—21.4)	21.6 (10.3—45.3)	25.4 (10.6—61.4)	96.8 (42.5—220.8)
24 h before vaccination	6	5.0 (3.4— 7.3)	10.8 (7.5—15.5)	16.3 (12.9—20.6)	20.5 (15.1—27.9)	103.5 (65.8—162.1)
Simultaneously with vaccination	6	5.8 (4.2— 8.0)	7.6* (4.7—12.3)	10.8 (5.4—21.4)	14.5 (6.8—30.9)	143.7 (83.3—248.6)
24 h after vaccination	6	5.8 (4.2— 8.0)	8.0* (5.1—12.7)	10.9 (5.8—21.0)	16.0 (8.4—30.5)	86.3 (52.4—142.5)
72 h after vaccination	6	5.7 (3.8— 8.4)	11.0 (8.3—14.6)	17.4 (10.1—29.8)	23.1 (12.0—44.8)	114.1 (55.7—232.2)
Unvaccinated control	6	4.6 (3.3— 6.4)	5.0** (3.6— 7.0)	5.7** (4.0— 8.2)	8.1*** (6.5—10.3)	64.0 (26.4—155.5)

* $P<0.05$.
** $P<0.01$ (Significance of difference from titres of pigs treated with vaccine alone).
*** $P<0.001$.

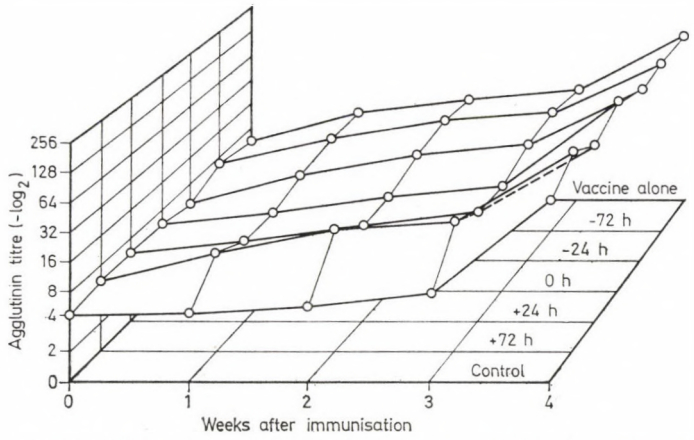

Fig. 4. Effect of Retardillin given at different dates compared to vaccination with swine erysipelas adsorbate vaccine on the culture agglutination test

A correlation was found between the intensity of cutaneous reaction to challenge, and the pre-challenge agglutinin titre of vaccines. Pigs having a reciprocal mean titre of 28.7 (95% fiducial limits: 22.5—36.5) proved to be immune, *i.e.* survived the challenge without any clinical change. The mean titre of partially immune pigs, responding to challenge by a slight cutaneous change, had been 16.9 (11.8—23.9), and that of susceptible pigs 7.5 (6.1—9.3). The latter animals developed a severe cutaneous reaction and a lasting febrile course indicative of bacteriaemia in response to challenge.

Discussion

Like mice in the model experiments, pigs showed depression of immune response to the live swine erysipelas vaccine VR_2 when treated with Retardillin. The degree of immunosuppression depended on the dose of the antibiotic, and on the time of its administration relative to vaccination as well.

Response of the pigs treated with VR_2 vaccine plus Retardillin to challenge, and serological examinations have unequivocally indicated that administration of the usual dose of antibiotic simultaneously with the vaccination unfavourably influence the immunity of the vaccines. Pigs treated with Retardillin 24 h after vaccination scarcely differed in response from non-vaccinees, and Retardillin administration 24 h before or 72 h after vaccination also had a notable immunosuppressive effect. It can be concluded that pigs must not be given antibiotic from day 1 before, to at least day 3 after, immunization with the live swine erysipelas vaccine VR_2.

Experiments on the influence of antibiotic treatment on immune response to an inactivated adsorbed swine erysipelas vaccine had inconsistent results in mice and pigs. This can be explained partly by the ten times lower number

of experimental pigs compared to mice, partly by marked species differences in reactivity. Furthermore, model experiments in mice have suggested that antibiotic influence on the mammalian immune system is of short duration, and reversible, having a major impact only if either the antibiotic dose is large or the antigenic stimulus low, or both conditions are simultaneously present. Elimination of the antibiotic from the organism is followed by gradual nor. malization of the immunological functions. This implication is based on the serological evidence of a marked antibody titre depression after large doses of Retardillin. Nevertheless, evaluation of titre differences between vaccinees treated and not treated with antibiotic is difficult if the adsorbed vaccine is used, for this stimulates antibody synthesis much slower and in a considerably lower concentration than the live VR_2 vaccine. The agglutinin titres of VR_2-vaccinees increased 8—17 times over the pre-vaccination level, depending on the vaccine dose, while those of the pigs treated with the adsorbed vaccine increased only 2—3 times.

Thus, unlike mice, pigs immunized with the inactivated adsorbed swine erysipelas vaccine showed no notable depression of immune response on treatment with Retardillin in the usual dose range (10,000—40,000 IU/kg bwt). A very high dose of the antibiotic (160,000 IU/kg bwt), however, accounted for a similar degree of immunosuppression as in mice. In view of this, it is recommended that pigs requiring for any reason a large-dose antibiotic treatment during the days preceding or succeeding immunization with an adsorbed swine erysipelas vaccine should be revaccinated after three weeks.

Summary

The influence of antibiotic treatment on immune response to live or inactivated swine erysipelas vaccine was examined in pigs.

Response to challenge with virulent *E. rhusiopathiae* bacteria three weeks after simultaneous treatment with live or inactivated vaccine plus rising doses of the antibiotic Retardillin (10,000—160,000 IU/kg bwt) differed between the two vaccinee groups. While the pigs immunized with the live VR_2 vaccine showed a dose-dependent decrease of immunity at all levels of antibiotic treatment except 10,000 IU/kg bwt, those given the adsorbed vaccine showed it only at the extreme level of 160,000 IU/kg bwt.

Serological tests disclosed a dose-dependent depression of the serum agglutinin titres by the antibiotic in both vaccinated groups, especially among the VR_2-vaccinees.

Treatment with an identical dose of antibiotic at different times relative to immunization showed that in VR_2-vaccinees Retardillin developed maximum immunosuppressive action on administration simultaneously with, or within 24 h before of after, vaccination, while in pigs given the adsorbed vaccine the simultaneous and 24 h postvaccination treatment were the most effective, antibiotic treatment 72 h before and after vaccination accounted for a negligible and minor immunosuppression, respectively.

These findings have been substantiated by serological assay of agglutinin titres.

The results of mouse and pig experiments equally support the conclusion that Retardillin treatment in the period of active immunization against swine erysipelas depresses the immune response to both live and adsorbed vaccines.

In practical terms, the efficiency of immunization with the live vaccine presupposes the complete withdrawal of antibiotics for the critical period, while with adsorbed vaccines only large doses are likely to interfere.

If, for any reason, antibiotic withdrawal is impossible, the vaccination should be repeated three weeks later.

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DIE HÄUFIGKEIT DER EXUDATIVEN (PSE) FLEISCHBESCHAFFENHEIT BEI EINIGEN HEIMISCHEN SCHWEINERASSEN

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Durch systematische Selektion wurde in den letzten Jahrzehnten das Fleischbildungsvermögen der Schweine beträchtlich gesteigert. Die angewandten intensiven züchterischen Methoden aber bedeuteten einen wesentlichen Eingriff in das genetische Gleichgewicht der einzelnen Populationen, was zur Entstehung stressempfindlicher Rassen führte. In diesem Zusammenhang hat die Verbreitung der PSE (»pale, soft, exudative«) Fleischbeschaffenheit immer größere Ausmaße angenommen.

Zahlreiche Wissenschaftler versuchten die Ursachen für das Auftreten von PSE-Fleisch aufzudecken. Nach (BENDALL u. Mitarb., 1962; BLENDL u. Mitarb., 1978; HAMM, 1969, 1977; HILDEBRANDT, 1974; KÖRMENDY, 1971; POTTHAST u. Mitarb., 1976; TAKÁCS u. Mitarb., 1978; WISMER-PEDERSEN u. Mitarb., 1961) laufen im PSE Fleisch die biochemischen Vorgänge der Glykolyse und des ATP Abbaus wesentlich rascher als in Muskeln normaler Fleischbeschaffenheit ab. Durch diesen schnellen Abbau sinkt der pH-Wert bereits in der ersten Stunde p. m. auf sehr niedrige Werte ab, was ein vermindertes Wasserbindungsvermögen und die blaße Farbe zur Folge hat.

Bei starken körperlichen Belastungen vor dem Schlachten der Tiere, zeigten stressempfindliche Rassen einen niedrigeren Muskel pH-Wert als stressresistente (ENDER u. Mitarb., 1974; HAVAS, 1977; LOSONCZYNÉ, 1967; PRANGE u. Mitarb., 1977; SCHEPER, 1972, 1977; TAKÁCS, 1971). Aus diesem Grunde versuchte man Verfahren zu entwickeln, die eine schnelle und sichere Bewertung der Fleischbeschaffenheit ermöglichen (FÉSÜS, 1978; HERRING u. Mitarb., 1971; HONIKEL u. Mitarb., 1977; SYBESMA u. Mitarb., 1978; WENIGER u. Mitarb., 1964).

Es zeigte sich eindeutig, daß zur Aufhebung der PSE Beschaffenheit, neben der Beachtung von entsprechenden schlachtungs- und haltungstechnologischen Faktoren nur die Züchtung stressresistenter Linien zu dauerndem Erfolg führen kann.

Da die Häufigkeit des Auftretens einer PSE-Fleischbeschaffenheit bei unseren Schweinerassen und Hybriden bisher nicht bekannt war, stellen unsere Untersuchungen einen wesentlichen Beitrag zur Klärung dieser Frage dar.

Untersuchungsmaterial und Methodik

Die Untersuchungen wurden in der Nachkommenprüfstation Kecskemét durchgeführt. Das Tiermaterial wurde entsprechend den Mastprüfungsnormen mit einer durchschnittlichen Körpermasse von 30 kg eingestallt und nach vollständiger Manifestation des genetischen Potentials bei ca. 103 kg Körpermasse geschlachtet. Da die Rasse und Abstammung der Tiere genau festgestellt wurde und die Haltungs- und Fütterungsbedingungen, sowie die Transport- und Schlachtbedingungen in den Untersuchungsgruppen gleichgehalten werden konnten, können die auftretenden Unterschiede in der Fleischqualität den genetisch bedingten Rassenunterschieden zugeschrieben werden. Da die Schweine unmittelbar vor dem Schlachten zwischen Stall und Schlachtbank mit einem Elektrokarren, nur etwa 100 Meter weit transportiert wurden, bedeutete der Transport praktisch keine Stresswirkung. Diese vorteilhaften Umstände bewogen uns, die Nachkommenprüfungstation in Kecskemét zum Durchführen der Untersuchungen zu wählen. Im Untersuchungszeitraum 1977/1978 untersuchten wir Muskelproben von insgesamt 366 Schweinen verschiedener Rassen. Die Auswahl des Untersuchungsmaterials erfolgte stichprobenartig so, daß die gewonnenen Ergebnisse nur eine Tendenz im Auftreten der PSE Häufigkeit erkennen lassen.

Die Bestimmung des pH-Wertes, welcher 45 Minuten nach dem Schlachtprozess an der linken Hälfte des Schlachtkörpers ermittelt wurde, erfolgte mit einem tragbaren Radelkis pH-Meter. Der pH-Wert wurde an zwei Muskeln gemessen: im m. longissimus dorsi in der Höhe der letzten Rippe und im m. semimembranaceus. Nachfolgend wurden denselben Muskeln zur Laboratoriumsuntersuchung Proben entnommen. Nach 24 Stunden wurde der pH-Wert in den Fleischproben wieder bestimmt. Als PSE Fleisch wurden diejenigen Proben betrachtet, deren pH-Wert schon 45 Minuten nach dem Schlachten unter 5,7 lag.

Die Konsistenz der Fleischprobe wurde im Laufe der Laboratoriumsuntersuchungen mit dem Höpplerschen Konsistometer bei Zimmertemperatur mittels einer 5 Minuten lang währenden Belastung von 3000 Pond bestimmt. Unseren Erfahrungen gemäß dringt die Messstange im Falle von PSE Fleisch weniger als 2,5 mm in das Fleisch ein.

Das Wasserbindungsvermögen der Fleischproben wurde mit dem Braunschweigschen Kompressorium bestimmt. 0,3 g des Fleisches wurden 5 Minuten lang mit einem Schleicher-Schüll Filterpapier No 1117 komprimiert. Die Fläche des vom Filterpapier aufgesogenen ausgepressten Fleischsaftes wurde planimetrisch bestimmt. Bei PSE Fleisch war diese größer als 10 cm^2.

Zur Bestimmung der Farbe der Muskeloberfläche verwendeten wir das Spektrophotometer Spekol (Zeiss) das mit einem Rd/O Remissiometer kombiniert war. Letzteres bestimmt die Intensität des von der Fleischoberfläche

reflektierten Lichtes bestimmter Wellenlänge. Die bestimmten Remissionswerte der Probe wurden auf eine $BaSO_4$ Standardplatte bezogen, deren Remissionswert gleich 100% war. Bei 530 nm zeigten die normalen Fleischproben Remissionswerte zwischen 8—18%, die PSE Fleische über 18%.

Untersuchungsergebnisse

Die Häufigkeit der PSE-Fleischbeschaffenheit der untersuchten Rassen ist in Tabelle I dargestellt. Tabelle II zeigt die Verteilung der PSE Fleischbeschaffenheit nach Rassen und Muskeln.

Tabelle I

Häufigkeit der PSE Fleischbeschaffenheit bei einzelnen Schweinerassen

Rasse	Anzahl der untersuchten Tiere	Anzahl der Tiere mit PSE Beschaffenheit	PSE Beschaffenheit in Prozent
Ungarisches großes weißes Fleischschwein	100	14	14
Schwedische Landrasse	70	20	28
Estnisches Landschwein	45	8	17
DDR Landrasse	38	12	31
Holländische Landrasse	27	6	22
Englische Landrasse	16	2	12
Kahyb Rasse	70	21	30
Insgesamt:	366	83	Durchschnitt: 22

Tabelle II zeigt die Verteilung der PSE Fleischbeschaffenheit nach Rassen und Muskeln.

Tabelle II

Die Häufigkeit der PSE Fleischbeschaffenheit in Beziehung der Rassen und Muskeln

Rasse	Anzahl von PSE Beschaffenheit-Fällen			Insgesamt	
	im Kotlett	im Schinken	in beiden Muskeln	Anzahl	Prozent
Ungarisches großes weißes Fleischschwein	11	2	1	14	14
Schwedische Landrasse	18	—	2	20	28
Estnisches Landschwein	6	1	1	8	17
DDR Landrasse	9	—	3	12	31
Holländische Landrasse	4	1	1	6	22
Englische Landrasse	1	1	—	2	12
Kahyb Rasse	15	4	2	21	30
Zusammen	64	9	10	83	Durchschnitt: 22

Gemäß Tabelle II war die Häufigkeit der PSE Beschaffenheit bei allen Rassen der untersuchten Population im Kotlett größer als im Schinken. Auf die gesamte untersuchte Population bezogen, betrug die Häufigkeit der PSE Beschaffenheit im Kotlett 78%, im Schinken 10%, in allen beiden Muskeln 12%.

Fleisch mit DFD (dunkel, fest, trocken) Merkmalen wurde bei unseren Untersuchungen nicht gefunden, was vermutlich darauf zurückzuführen war, daß die Tiere ohne Transportbelastung, in ausgeruhtem Zustand geschlachtet wurden und somit ihr Glykogenvorrat nicht verbraucht wurde.

Diskussion

Die Ergebnisse unserer Untersuchung beweisen, daß die PSE-Fleischbeschaffenheit auch bei unseren heimischen Schweinerassen auftritt. In der PSE Häufigkeit der von uns untersuchten Rassen waren bedeutende Unterschiede vorhanden, besonders auffallend war die Differenz zwischen dem ungarischen weißen Fleischschwein (14%) und der DDR Landrasse (31%). Eine besondere Aufmerksamkeit verdiente die Erfahrung, daß auch in dem als stressresistent bekannten ungarischen weißen Fleischschwein und dem estnischen Landschwein die PSE Beschaffenheit, wenn auch weniger häufig aber doch vorkam. Diese Erscheinung kann mit großer Wahrscheinlichkeit darauf zurückgeführt werden, daß bei der züchterischen Bestrebung, das Fleisch-Fett-Verhältnis zu verbessern, andere Gesichtspunkte weniger beachtet wurden.

Das gehäufte Auftreten der PSE-Fleischbeschaffenheit und dessen Merkmalen bei den übrigen von uns untersuchten Rassen überraschte uns nicht, da sich die Ergebnisse mit den in der Literatur beschriebenen Häufigkeiten anderer in der Fleischproduktion führender Länder Europas decken.

Unseren gegenwärtigen genetischen Kenntnissen gemäß stehen die zur Auflösung des Widerspruches »größere Fleischproduktivität–mindere Fleischqualität« geeignete Mittel uzw. Kreuzung stressresistenter Rassen, Halothantest, Blutgruppen und enzympolymorphische Untersuchungen usw. zur Verfügung. Um die Wirtschaftlichkeit der gesamten Schweinezucht nicht weiter einzuschränken und um die Fleischqualität zu erhöhen, wird es notwendig sein, die erwähnten Teste, möglichst bald und in breitem Umfange anzuwenden.

Zusammenfassung

An 366 Schlachtschweinen verschiedener Rassen einer Nachkommenprüfstation wurde die Häufigkeit und das Auftreten von PSE-Fleischbeschaffenheit in Ungarn untersucht. Das Tiermaterial wurde unter gleichen Bedingungen gehalten, gefüttert und geschlachtet. Die Parameter pH-Wert, Muskelfarbe, Konsistenz und das Safthaltevermögen wurden an den

m. longissimus dorsi und m. semimembranaceus bestimmt. Zu den Muskelproben der zufällig ausgewählten Tiere kam im Populationsdurchschnitt eine PSE Fleischbeschaffenheit von 22% vor. In dem ungarischen großen weißen Fleischschwein zeigte sich diese Fleischbeschaffenheit in einer Häufigkeit von 14%, in der DDR Landrasse in einer solchen von 31%. In der schwedischen, estnischen, holländischen, englischen Land- und der »Kahyb«-Rasse hing die Häufigkeit der PSE-Veränderung von der Intensität der Fleischproduktivität ab. Die Häufigkeit der PSE Veränderung betrug — alle Fälle in Betracht gezogen —, in dem langen Rückenmuskel 78%, in Schenkelmuskel 10%, in beiden Muskeln 12%.

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VARIA

World Association for the Advancement of Veterinary Parasitology Nineth International Conference 14th—17th July 1981. Budapest, Hungary

The above Conference will be organized by the Hungarian Society of Parasitologists at the University of Veterinary Science of Budapest.

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Large scale management systems and parasite populations.
Ecologal basis of parasite control,
Advances in animal parasitology (free topics).

Poster sessions and an exhibition of pharmaceutical products will also be organized.

Further informations can be obtained from the secretaries of national parasitological organizations or from the secretary of the Organizing Committee:

Dr. T. Kassai, Secretary
9th International Conference of the W.A.A.V.P.
University of Veterinary Science
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H—1077 Budapest

INDEX

Bacteriologia

GERESSU, B. and TAKÁCS, J.: Identification of Genera in the Family of *Enterobacteriaceae* by a U-tube Multitest Method in Food Microbiology 241

Virologia

BODON, L. and PROHÁSZKA, L.: Isolation of an Adenovirus from Rabbits with Diarrhoea 247

MOCSÁRI, E. and HORVÁTH, Irén: Properties of the "CKp" Strain of Transmissible Gastroenteritis Virus. II. Studies on the *in vivo* Viral Replication in Newborn Piglets .. 257

Immunologia

SEMJÉN, G. and PESTI, L.: LT Antitoxin Titres in Three-Week-Old, *E. coli*-Vaccinated Pigs 269

PADÁNYI, M., RÉTHY, L., KULCSÁR, Á., GÉRESI, Magdolna, GELENCSÉR, F., POGÁNY, I., BÁCSKAI, L. and RÉTHY, L. A. Jr.: The Development of Primary Antibacterial Immune Protection against *Erysipelothrix rhusiopathiae* and the Effect of Anaerobic Corynebacterial Immunostimulants (Short communication) 273

RÉTHY, L., GÉRESI, Magdolna, GELENCSÉR, F., ÉCSY, Rita, PADÁNYI, M., POGÁNY, I., BÁCSKAI, L. and RÉTHY, L. A. Jr.: Heat-Inactivated *Brucella* Suspensions as Modulators of Hosts' Defence against Tumour (Short communication) 277

GÉRESI, Magdolna, RÉTHY, L., ÉCSY, Rita, GELENCSÉR, F., PADÁNYI, M., POGÁNY. I., BÁCSKAI, L. and RÉTHY, L. A. Jr.: Antitumour Effect of Different Corynebacterial Immunostimulants in Non Isogenic Mouse-Tumour-System (Short communication) 281

GELENCSÉR, F., RÉTHY, L., BÁCSKAI, L., GÉRESI, Magdolna, POGÁNY, I., PADÁNYI, M. and RÉTHY, L. A. Jr.: The Effect of Anaerobic Corynebacterial Immunostimulants on the Primary Immune Response to *Tetanus* Toxoid and *Perfringens* Epsilon Toxoid (Short communication) 285

SÓLYOM, F., FAZEKAS, Á. and CZELLENG, F.: Storage-Related Potency Changes in Monovalent Type O_1, A_5 and C Foot-and-Mouth Disease Vaccine Preparations 289

TRAN DIHN TU: Effects of Antibiotics on the Immune System of Animals. I. Mouse Experiments with the Low-Virulence VR_2 Strain of *Erysipelothrix rhusiopathiae* 297

TRAN DIHN TU: Effects of Antibiotics on the Immune System of Animals. II. Mouse Experiments with an Inactivated Adsorbed Vaccine against Swine Erysipelas ... 309

TRAN DIHN TU: Effects of Antibiotics on the Immune System of Animals. III. Experiments in Pigs Immunized with Live and Inactivated Swine Erysipelas Vaccines 317

Physiologia

KELLNER, Ágnes, SÁNDOR, I. und TAKÁCS, J.: Die Häufigkeit der exudativen (PSE) Fleischbeschaffenheit bei einigen heimischen Schweinerassen 333

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ACTA VETERINARIA

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РЕЗЮМЕ

ИДЕНТИФИКАЦИЯ РОДОВ СЕМЕЙСТВА ENTEROBACTERIACEAE МУЛТИТЕСТ МЕТОДОМ ПРИ ПОМОЩИ U-ПРОБИРКИ В МИКРОБИОЛОГИИ ПИЩЕВЫХ ПРОДУКТОВ

Б. ГЕРЕССУ и Я. ТАКАЧ

Разработан модифицированный метод дифференциации родов семейства **Enterobacteriaceae** при помощи новой политропной среды в U-пробирке. Среда, содержащая компонент Вож-Прокауер, показывает расщепление H_2S и индола, подвижность и активность лизин-декарбоксилазы. Метод лучше реакций IMVIC для дифференциации друг от друга родов **Escherichia, Citrobacter, Salmonella-Arizona, Enterobacter, Hafnia, Klebsiella.** Этот способ дифференциации быстр, дешев и легко применим. Результаты воспроизводимы.

ИЗОЛЯЦИЯ И ИДЕНТИФИЦИРОВАНИЕ АДЕНОВИРУСА ИЗ КРОЛИКОВ С ПОНОСОМ

Л. БОДОН и Л. ПРОХАСКА

В одной части кроликов крупнохозяйственного поголовья с поносом — особенно в начале клинических признаков — авторы обнаруживали жидкое содержимое в слепой кишке, в котором низкое число кишечной палочки только позже поднялось до характерного для заболевания уровня. Из органов, кишечной стенки и кишечного содержимого изолировали аденовирус. Изолированный вирус размножался только в гомологической тканевой культуре; в изученных гетерологических культурах размножения не было. В первичной тканевой культуре из почки кролика, вызванные вирусом ядерные включения Коудри типа А замечались только после окрашивания. Цитопатогенного действия после первых пассажей ни макроскопически, ни микроскопически не наблюдали. Вирус содержал ДНК, был резистентным к хлороформу и трипсину, выдержал воздействие pH 3,0 и температурой. Скорость осаждения вируса равнялась 1,32 г/мл, а его размер — 80 нм-ам. Вирус не нейтрализировали антисыворотки против аденовирусов свиньи или крупного рогатого скота, ни таковые против человечьих аденовирусов, полученные из лошади. Он нейтрализировался только гиперимунными антисыворотками из кроликов и морских свинок, зараженных изолированным вирусом. В РСК с антигексоновыми сыворотками против человечьих серотипов и изолированным вирусом обнаружен общий комплементсвязывающий антиген титра 1 : 40 — 1 : 80. Частичная идентичность антигена доказана появлением шпор в пробе агардиффузионной преципитации с вышеуказанными веществами.

Антитела в сыворотке 5—7-недельных кроликов обнаруживались только в низких титрах или их совсем не было. Но один месяц позже в образцах сыворотки от выживших кроликов антитела уже налицевствовали даже в высоких титрах (1 : 10 — 1 : 100). В сыворотке более пожилых кроликов уровень количества антител уже был высоким (1 : 100). На основании полученных результатов авторами поднимается вопрос о возможной роли аденовируса в патогенезе поноса кроликов.

Это первый случай изоляции аденовируса из кролика, поэтому авторы предлагают международному коммитету номенклатуры включить его в список аденовирусов под названием RA-1 или аденовирус г 1.

СВОЙСТВА ШТАММА СКр ВИРУСА ТГЭ

II. ИЗУЧЕНИЕ РЕПЛИКАЦИИ ВИРУСА В НОВОРОЖДЕННЫХ ПОРОСЯТАХ

Е. МОЧАРИ и ИРЕН ХОРВАТ

Методом иммунофлуоресценции (ИФ) авторами изучалось размножение штамма СКр вируса ТГЭ *in vivo* в новорожденных поросятах. Эти наблюдения дополнили микроскопическим и электронномикроскопическим изучением тонкого отдела кишечника и реизоляцией вакцинного вируса из кишки.

Обнаружено, что перорально введенный вакцинный вирус размножается в первую очередь в тонком отделе кишечника, кроме этого в легких и тонзиллах. Доказано, что репликация вируса осуществляется на всем протяжении тонкого отдела кишечника. Электронно-микроскопическим исследованием доказано наличие вируса в протоплазме дифференцированных эпителальных клеток кишечника и он реизолировался из тонких кишек на тканевых культурах.

Нормальным микроскопом не обнаружено изменений ни в эпителиальных клетках, прокрывающих кишечные ворсинки, ни таковых, выстилающих Либеркюновы крипты.

ТИТРЫ АНТИТОКСИНА ПРОТИВ ТЕПЛОЧУВСТВИТЕЛЬНОГО ЭНТЕРОТОКСИНА У ТРЕХНЕДЕЛЬНЫХ ПОРОСЯТ, ВАКЦИНИРОВАННЫХ ПРОТИВ КИШЕЧНОЙ ПАЛОЧКИ

Г. ШЕМЙЕН и Л. ПЕШТИ

Парэнтеральной иммунизацией 22-дневных поросят комплексной, формалином инактивированной вакциной из кишечной палочки согласно методу Касаи и Боровс, удалось добиться примерно четырехкратного увеличения уровня антитоксина против теплочувствительного энтеротоксина в сыворотке животных.

ВЫРАБОТКА «ПЕРВИЧНОГО» ИММУНИТЕТА ПРОТИВ ERYSIPELOTHRIX RHUSIOPATHIAE ПРИ ВОЗДЕЙСТВИИ ИММУНОСТИМУЛЯНТОВ ИЗ АНАЭРОБНЫХ КОРИНЕБАКТЕРИЙ

М. ПАДАНИ, Л. РЕТИ, А. КУЛЧАР, МАГДОЛНА ГЕРЕШИ, Ф. ГЕЛЕНЧЕР, И. ПОГАНЬ, Л. БАЧКАИ и Л. А. РЕТИ (мл.)

На мышах изучалась выработка «первичного» иммунитета при воздействии иммуностимулянтов из анаэробных коринебактерий после применения осадочной противорожистой вакцины.

При одновременном применении иммуностимулянтов с иммунизирующим антигеном обнаружено, что они усиливают напряженность формирующегося иммунитета. Если эти вещества применялись до введения антигена, то они проявляли иммуносупрессивный эффект. Если они применялись после введения антигена, то этот факт проявлялся — в зависимости от применявшихся штаммов или в иммуносупрессивном действии (**C. parvum RR-1, C. parvum RR-12**), или не сказывался на выработке иммунитета (**C. acnes RR-2, C. lymphophylum RR-33, C. granulosum RR-H5**).

ПОВЫШЕНИЕ АНТИОПУХОЛЕВОЙ ЗАЩИТНОЙ СПОСОБНОСТИ ОРГАНИЗМА ПРИ ПОМОЩИ БРУЦЕЛЛЕЗНОЙ КУЛЬТУРЫ, ИНАКИВИРОВАННОЙ ТЕМПЕРАТУРОЙ

Л. РЕТИ, МАГДОЛНА ГЕРЕШИ, Ф. ГЕЛЕНЧЕР, РИТА ЭЧИ, М. ПАДАНИ И. ПОГАНЬ, Л. БАЧКАИ и Л. А. РЕТИ (мл.)

Авторами изучалось противоопухолевое действие бруцеллезных культур, инактивированных температурой на неизогенной мышиной модели с асцитной опухолью Эрлиха. На основании экспериментов установлено, что иммуностимулянты, приготовленные из

штаммов РН-0159, РН-0260 и РН-0371 **Brucella abortus** сигнификантно продлили выживаемость пораженных опулью Эрлиха мышей по сравнению с контрольными таковыми. Инактивированная суспенсия штамма РН-0482 **Brucella suis** не усилила устойчивость организма к опухоли.

ПРОТИВООПУХОЛЕВОЕ ДЕЙСТВИЕ ИММУНОСТИМУЛЯ НТОВИЗ АНАЭРОБНЫХ КОРИНЕБАКТЕРИЙ В НЕИЗОГЕННОЙ СИСТЕМЕ МЫШЬ-ОПУХОЛЬ

МАГДОЛНА ГЕРЕШИ, Л. РЕТИ, РИТА ЭЧИ, Ф. ГЕЛЕНЧЕР, М. ПАДАНИ, И. ПОГАНЬ, Л. БАЧКАИ и Л. А. РЕТИ (мл.)

Изучено антипухолевое действие иммуностимулянтов из разных коринебактерий в неизогенных мышах, пораженных опухолью асцитес Эрлиха. Изучалась и взаимосвязь между дозой иммуностимулянтов и степенью антиопухолевого действия. Данные экспериментов показали, что все применявшиеся суспенсии коринебактерий продлили выживаемость обработанных животных. Иммунопотенциаторы, приготовленные из штаммов **C. parvum, C. acnes** и **C. granulosum** уже в малых дозах (8 О. Е.) давали выраженный антиопухолевый эффект. Зато иммуностимулянт из **C. lymphophylum** давал антиопухолевый эффект только при повышенных (20 О. Е.) дозах. Антиопухолевый эффект иммуностимулянта из **C. avidum**, независимо от дозы, был одинаковым.

ДЕЙСТВИЕ ИММУНОСТИМУЛЯТОРОВ ИЗ КОРИНЕБАКТЕРИЙ НА ФОРМИРОВАНИЕ ПЕРВИЧНОГО ИММУНИТЕТА ПРОТИВ ТОКСОИДОВ ТЕТАНУСА И ПЕРФРИНГЕНС ЭПСИЛОН

Ф. ГЕЛЕНЧЕР, Л. РЕТИ, Л. БАЧКАИ, МАГДОЛНА ГЕРЕШИ, И ПОГАНЬ, М. ПАДАНИ, и Л. А. РЕТИ (мл.)

Авторами изучалось формирование выносливости к токсину у мышей, иммунизированных токсоидами из тетануса и перфрингенс эпсилон, при одновременном применении иммуностимулянта, приготовленного из анаэробных коринебактерий. Обнаружено, что иммунитет к токсоиду перфрингенс эпсилон усиливает только иммуностимулянт из **C. parvum** штамм RR-1, тогда как суспенсия из других штаммов вызывает по сути иммуносупрессивный эффект. На эффективность иммунизации токсоидом из тетануса иммуностимулянты, приготовленные из штаммов **C. parvum RR-12** и **C. acne RR-2**, не действовали. Но таковые из других штаммов усиливали иммунитет, возникающий от действия токсоида тетануса.

ХРАНЕНИЕМ ОБУСЛОВЛЕННЫЕ ИЗМЕНЕНИЯ ИММУНИЗИРУЮЩЕЙ СПОСОБНОСТИ ПРОТИВОЯЩУРНЫХ МОНОВАЛЕНТНЫХ ВАКЦИН ТИПА O_1, A_5 и C

Ф. ШОЙОМ, А. ФАЗЕКАШ и Ф. ЦЕЛЛЕНГ

Всего 52 противоящурных моновалентных вакцины типа O_1, A_5 и C, приготовленные процедурой Вальмана, изучались на имунизирующую способность в течение трехлетнего хранения. Эксперименты определения иммунизирующей способности хранившихся вакцин осуществлялось на морских свинках методом индекса С и на половозрелых мышах методом индекса Е. Вакцины изучались неделю после их приготовления и после разных сроков хранения, применяя всегда тот-же образец. Упомянутыми двумья методами определены следующие сроки годности:

Индексы	Типы вакцин		
	O_1	A_5	C
C	23 месяца	20 месяцев	32 месяца
E	24 месяца	14 месяцев	29 месяцев

Не было существенной разницы в иммунизирующей способности отдельных вакцин после одного года хранения, вакцины типа С обладали еще удовлетворительными иммуногенными свойствами даже после двух лет хранения. Иммуногенные свойства наибыстрее ухудшились у вакцин типа A_5, у вакцин же типа O_1 ухудшение наступило позже, а у таковых типа С — наипозже. Эта последовательность в сроке годности является средним показателем данного типа, ибо внутритиповая изменчивость была довольно большой (от 10 до 38 месяцев). Содержание антигена в вакцинах, выраженное в единицах CF, колебалось в данных пределах в зависимости от pH. Между уровнем свободного формалина и сроком годности никакой взаимосвязи не обнаружено. На основании данных наблюдений одногодичный срок годности, определенный для вакцин типа Вальдмана, является реальным, но целесообразно разные образцы время от времени на годность проверить.

ДЕЙСТВИЕ АНТИБИОТИКОВ НА ФОРМИРОВАНИЕ ИММУНИТЕТА В ОРГАНИЗМЕ ЖИВОТНЫХ

I. ЭКСПЕРИМЕНТЫ НА МЫШАХ С ОСЛАБЛЕННОЙ ВИРУЛЕНТНОСТИ РОЖИСТЫМ ШТАММОМ VR_2

ТРАН ДИН ТУ

Согласно общепринятому воззрению (хотя литературные данные в этом отношении частично противоречивы) одновременное применение антибиотиков искажает иммуногенное действие вакцин, приготовленных из бактерий ослабленной вирулентности или из авирулентных таковых.

Ради разъяснения данного вопроса автором проведены модельные эксперименты иммунизации мышей противорожистой вакциной из штамма VR_2. Получены следующие результаты.

Вакцинный штамм VR_2 при сравнении с вирулентным рожистым штаммом ОК 22 показывал *in vitro* такую же чувствительность к пенициллину. *In vivo* защитный эффект ретардилина у мышей при той же дозе антибиотика зависит главным образом от количества введенных микробов.

Прочность иммунитета от вакцинного штамма VR_2 защищает мышей от последствий суперинфекции две недели спустя 1000 LD_{50} вирулентного штамма в зависимости от дозы вакцины (количества введенных микробных телец): введение вакцины, содержащей 10^2 микробных телец обеспечивает только 10,37%-ную выживаемость, тогда как вакцина с 10^5 микробными тельцами — 96,67%-ную выживаемость. Защитный показатель, выраженный процентом выживаемости, показывает положительного коэффициента линейную взаимосвязь с логарифмически выраженным количеством микробных телец в вакцине. Дальнейшее повышение количества микробов в вакцине тормозится резидуальной вирулентностью штамма VR_2 к мыши.

Процент выживаемости мышей, иммунизировавшихся вакциной VR_2 и одновременно обработавшихся повышенными дозами ретардиллина после реинфекции две недели спустя 1000 LD_{50} зависит отчасти от дозы вакцины (количества введенных микробных телец), отчасти от дозы антибиотика. Защитный эффект, выраженный процентом выживаемости, показывает экспоненциальную взаимосвязь отрацательного коэффициента с дозой ретадиллина, выраженной М. Е./кг живого веса.

При пользовании данной дозой ретардиллина (40 000 М. Е./кг живого веса) эффективность иммунизации вакциной VR_2 зависит и от того, какой срок времени истек от вакцинации до введения антибиотика. Более отрицательный эффект оказывало введение антибиотика сутки спустя, чем сутки до вакцинации. Введение ретардиллина сутки до или трое суток после вакцинации уже не сказывалось отрицательно на формировании иммунитета. Вакцинная доза сильно сказывается на выработке иммунитета.

ДЕЙСТВИЕ АНТИБИОТИКОВ НА ФОРМИРОВАНИЕ ИММУНИТЕТА В ОРГАНИЗМЕ ЖИВОТНЫХ

II. ЭКСПЕРИМЕНТЫ НА МЫШАХ С ИНАКТИВИРОВАННОЙ ОСАДОЧНОЙ ПРОТИВОРОЖИСТОЙ ВАКЦИНОЙ

ТРАН ДИН ТУ

Автором изучалось действие одновременно введенного антибиотика на иммунизирующую способность осадочной вакцины против рожи свиней.

Серией экспериментов на мышах доказано, что у животных, иммунизированных противорожистой осадочной вакциной при одновременном введении повыщающихся доз ретардилина, защита против 1000 LD_{50} заражения вирулентным штаммом рожи свиней соразномерно дозе антибиотика понизилась. Это понижение защищенности при введении 20 000, 40 000 и 80 000 М.Е./кг живого веса ретардиллина равнялось 29,5, 58,2 и 73,9%-ам соответственно по сравнению с только вакцинировавшимися мышами.

Таким образом одновременное введение антибиотика отрицательно сказывается на иммунизирующем действии антигена не только из живых, но и убитых микробов. Это иммуносуппрессивное действие соразмерно дозе антибиотика. Защитная степень вакцины, выраженная в иммунологических единицах, показывает экспоненциальную связь отрицательного коэффициента с дозой ретардиллина, выраженной в М. Е./кг живого веса.

Иммуносупрессивное действие введения антибиотика и при пользовани осадочной противорожистой вакциной зависит от времени, истекшего после вакцинации, как это наблюдалось и в экспериментах с предыдущей противорожистой вакциной VR 2. Соответствующая доза ретардиллина (80 000 М. Е./кг) при одновременном введении и с вакциной вызывала наиболее интенсивное иммуносупрессивное действие. Понижение защитного эффекта по сравнению с только иммунизировавшимися мышами равнялось 69,1%-ам. При введении антибиотика сутки до и сутки и трое суток после вакцинации наблюдалось 29,5, 57,5 и 33,4%-ное соответственно иммуносупрессивное действие.

Проведенные эксперименты являются доказательством того, что введение ретардиллина вызывает переходное повреждение иммунологического аппарата организма и при пользовании осадочной противорожистой вакцины.

ДЕЙСТВИЕ АНТИБИОТИКОВ НА ФОРМИРОВАНИЕ ИММУНИТЕТА В ОРГАНИЗМЕ ЖИВОТНЫХ

III. ЭКСПЕРИМЕНТЫ НА СВИНЬЯХ С ПРОТИВОРОЖИСТОЙ ВАКЦИНОЙ ОСЛАБЛЕННОЙ ВИРУРЛЕНТНОСТИ VR_2 И ИНАКТИВИРОВАННОЙ ОСАДОЧНОЙ ТАКОВОЙ

ТРАН ДИН ТУ

После экспериментов, изложенных в первых двух сообщениях, автором изучалось формирование иммунитета у свиней от живой и от инактивированной противорожистой вакцины под воздействием ретардиллина.

Экспериментальные данные показали, что есть разница в прочности иммунитета от вакцины VR_2 и инактивированной осадвочной таковой при одновременном введении повышающихся доз ретардиллина (10 000—160 000 М. Е./кг) к реинфекции скарификацией вирулентного рожистого штамма три недели спустя.

Иммунизирующий эффект вакцины VR_2 ретардиллин — соразмерно дозе — всегда существенно снизил, тогда как иммунитет от инактивированной осадочной вакцины искажался только от высоких доз (160 000 М. Е./кг) ретардиллина.

Однако результаты серологического изучения поголовья при помощи реакции агглютинации показали, что одновременно введенный ретардиллин в зависимости от дозы существенно снизил титры антител как после вакцинации вакциной VR_2, так и после вакцинации инактивированной таковой. Здесь тоже от вакцины VR_2 получен более слабый иммунитет.

Эффективность вакцинации вакциной VR_2 наиболее искажался, если ретардиллин вводили одновременно или одни сутки до или одни сутки после вакцинации, тогда как напряженность иммунитета от инактивированной осадочной вакцины — если ретардиллин вводили одновременно или одни сутки после вакцины. Напряженность иммунитета снижающий эффект ретардиллина, если пользовались им трое сутки до вакцинации, был совсем незначительным, а при пользовании им трое сутки после вакцинации — только слабым.

Таким образом данные экспериментов на свиньях подтвердили правильность наблюдений на мышах. При иммунизации против рожи свиней, если будут одновременно пользоваться ретардиллином, может наступить иммуносупрессивный эффект независимо от того, какой вакциной пользовались, то-ли живой, ослабленной вирулентности вакциной VR_2, или инактивированной осадочной таковой.

Из данных описанных экспериментов можно сделать тот практический вывод, что в дни пользования особенно живой, ослабленной вирулентности противорожистой вакциной

целесообразно воздержаться от пользования антибиотиком. При пользовании инактивированной осадочной вакциной только высокие дозы ретардиллина сказываются отрицательно на формировании иммунитета.

В данных случаях рекомендуется три недели спустя вакцинацию повторить, чтобы у животных выработался желаемый иммунитет.

ЧАСТОТА ОБНАРУЖИВАЕМОСТИ ТАК НАЗ ЭКССУДАТИВНОГО МЯСА У НЕКОТОРЫХ ВЕНГЕРСКИХ СВИНЫХ ПОРОД

А. КЕЛЬНЕР, И. ШАНДОР и Я. ТАКАЧ

На 366 свиньях разных пород на одной станции по контролю потомства изучалась частота обнаруживаемости так наз. экссудативного мяса. Животные содержались, кормились и их убой производили одинаково. На мышцах **m. longissimus dorsi** и **m. semimembranaceus** определяли pH, окраску, консистенцию и сочность мяса. В популяции зарегистрирована 22%-ная частота обнаруживаемости так наз. экссудативного мяса. Среди свиней венгерской большой белой мясной породы и ландрасской таковой из ГДР частота обнаруживаемости так наз. экссудативного мяса равнялась 14 и 31%-ам соответственно. Среди животных шведской, эстонской, голландской, английской ландрасс и породы кахиб частота обнаруживаемости так наз. экссудативного мяса зависела от интенсивности продукции мяса. Изменения наичаще обнаруживались в длинной спинной мышце (78%) и бедренной таковой (10%); в обеих мышцах данное изменение налицевствовало в 12%-ах случаев.

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PHYSICOCHEMICAL PROPERTIES OF FIELD AND CELL CULTURE-ATTENUATED STRAINS OF SWINE TRANSMISSIBLE GASTROENTERITIS (TGE) CORONAVIRUS

By

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(Received January 23, 1980)

Transmissible gastroenteritis (TGE) of swine differs from most of the known infectious diseases in the mechanisms of immunity. The essence of both the active and passive immunity is protection of the villous epithelial cells of the small intestine against TGE viral infections. An effective active immunity to TGE needs either oral infection with field strains of TGE virus (TGEV) or by oral vaccination with live attenuated TGEV. The use of live modified virus for developing live virus vaccines requires more detailed information on the differences existing between field and cell culture-attenuated TGEV strains. Although it is known that *in vivo* biological differences exist between virulent and cell culture-attenuated strains, the *in vitro* properties of these strains have been less explored. The present paper is a report on the comparison of selected physicochemical properties of three field strains and four cell culture-attenuated strains of TGEV, including the Hungarian "CKp" vaccine strain, with the objective of using them as possible *in vitro* markers.

Materials and methods

Cell cultures. Secondary swine thyroid (SSth) cell cultures were used throughout the experiments.

Virus strains. All the TGEV strains were plaque-purified three times in succession before tested. The following strains were employed. Field strains: the 9th-passage BB strain kindly supplied by J. BENYEDA (Budapest, Hungary), and the 11th-passage ESZT and the 5th-passage FC strains, both isolated in this laboratory. Cell culture-attenuated strains: the Hungarian "CKp" vaccine strain (CSONTOS and SZENT-IVÁNYI, 1971), the FS 216/64 strain (CARTWRIGHT et al., 1965), the Hannover strain (kindly supplied by K. H. WITTE, Hannover, W. Germany) and the Purdue strain (HAELTERMAN and

PENSAERT, 1967). The "CKp" strain had been passaged 15 times in swine kidney cell cultures and another 30 times in SSth cell cultures. The remaining strains had been passaged more than 200 times in SSth cell cultures after arrival at this laboratory. All the attenuated strains could be serially passaged at suboptimal temperature (28 to 33 °C) and produced significantly smaller plaques at supraoptimal temperature (41 °C) than the virulent strains did (BENYEDA, 1975).

Stocks of viruses were prepared in SSth cells. The maintenance medium consisted of Hanks' balanced salt solution (HBSS) enriched with 0.5% lactalbumin hydrolysate. The cell-associated virus was released by two rapid freeze-thaw cycles and the cell debris were removed by centrifugation at 3000 × *g* for 30 min. The supernatant fluid was filtered through a 450 nm Millipore filter and stored in 2 ml portions at —20 °C until tested.

In the chloroform, sodium desoxycholate and saponin resistance tests, a virulent strain of Aujeszky's disease virus, isolated at this laboratory, and the 5-DVIII strain of porcine enterovirus (SZENT-IVÁNYI, 1970) were included.

Virus titration. Serial 10-fold dilutions in amounts of 0.1 ml were inoculated into four SSth tube cultures per dilution. The titres ($\log_{10}$ $TCID_{50}$/0.1 ml) were calculated according to the method of KÄRBER (1931).

Chloroform sensitivity test. The original method was applied that had been described by PYL in 1951. Controls consisted of TGEV suspensions in which 20% phosphate-buffered-saline (PBS, pH 7.2) replaced the chloroform.

Treatment with sodium deoxycholate. The undiluted test virus was mixed with an equal volume of an 0.2% solution of sodium deoxycholate. Control virus was mixed with an equal volume of PBS. The mixtures were incubated either in a 37 °C water bath for one hour or at +4 °C for 16 hrs. The residual virus was titrated. The accuracy of the temperature in the water bath was assured by using a Heidolph T 51 immersion thermostat with a circulation pump.

Saponin-sensitivity test. 0.1 ml of a saponin solution (1 g of saponin [Merck] in 20 ml of HBSS) was added to 4.9 ml of the undiluted test virus. The mixture and the undiluted control virus were incubated at +4 °C for 20 hrs and, subsequently, its infectivity was titrated.

Trypsin resistance test. The test was performed according to the method of FURUUCHI et al. (1975). The test virus was diluted 10-fold with PBS. Then an equal volume of the diluted virus was mixed with a 1% solution of trypsin (DIFCO 1 : 250) in PBS, thoroughly shaken, and incubated at 37 °C. At the time the test began and 30, 60, 90, 120 min later, aliquots of virus from each test were mixed with equal volumes of HBSS containing 50% fetal calf serum and replaced in an ice bath. The HBSS was cooled to +4 °C before used.

Polyethylene glycol (PEG) sensitivity test. The original method of LOKTYEVA et al. (1973) was applied that had been developed to differentiate of

virulent and attenuated strains of type-1 poliovirus. SSth cells were grown in Petri dishes 40 mm in diameter. The plaque technique was the same as previously described (MOCSÁRI, 1980). The effect of PEG on the virus was determined by adding PEG (MW 40 000) to the agar overlay in a final concentration of 2%. The plaque production was compared with that obtained in cultures under agar overlay of conventional composition. The inhibiting effect of PEG on the plaque production was expressed in per cent.

Thermal-sensitivity test. The test virus was mixed with an equal part of distilled water and placed in a 50 °C water bath. At the time the test began and 2, 5, 10, 30 and 60 min later, the residual virus was titrated.

Cationic-stability test. A 2 *M* solution of $MgCl_2$ and $MgSO_4$ was mixed with an equal volume of undiluted test virus. The mixture was incubated in a 50 °C water bath. At the time the incubation began and 2, 5, 10, 30 and 60 min later, the virus infectivity was assayed.

pH-stability test. The test virus was diluted 10-fold in McIlvaine's buffer of pH 3.0 or 6.8. The pH values were measured before, during, and after the experiment. The mixtures were placed into a 37 °C water bath. At the time the incubation began and 2, 4, 6, 8 and 12 hrs thereafter, aliquots of virus from each test were placed in an ice bath, diluted 10-fold with Earle's balanced salt solution (pH 7.2, cooled down to +4 °C) and the infective virus was assayed.

All the above tests were carried out in duplicate experiments under identical circumstances. The results represent the arithmetical mean of the identical tests. For each selected property, all the TGEV strains were tested simultaneously.

Results

The infectivity of all TGEV strains was destroyed by adding chloroform in a final concentration of 20%.

All the strains were sensitive to the action of sodium deoxycholate in a final concentration of 0.1% (Table I). The infectivity of the "CKp" vaccine strain decreased by 3.25 $\log_{10}$ $TCID_{50}$ after 60 min at 37 °C. The remaining virus strains were either completely lost their infectivity within 60 min (Purdue and BB) or — varying each strain — a 2.75 to 4.87 $\log_{10}$ $TCID_{50}$ loss of virus infectivity was recorded.

Treatment with sodium deoxycholate at +4 °C reduced the infectivity of the "CKp" TGEV strain by 1.50 $\log_{10}$ $TCID_{50}$ in 16 h. The attenuated strain Purdue was completely inactivated, whereas the virulent, low-passaged strain FC was practically unaffected. The remaining TGEV strains showed a 1.75 to 3.50 $\log_{10}$ $TCID_{50}$ loss of infectivity (Table I).

Table I

Sensitivity of different TGEV strains to 0.1% sodium desoxycholate and 0.1% saponin

Treatment	TGEV strains							Control viruses	
	CKp	FS 216/64	Hanno-ver	Purdue	BB	ESZT	FC	Aujesz-ky	PEV**
Control	5.08*	5.83	5.08	5.42	4.87	4.56	5.12	6.50	7.00
Na-desoxycholate (37 °C, 60 min)	1.83	1.92	0.42	0	0	1.25	2.37	0	7.00
Control	5.63	6.00	5.00	5.12	4.62	4.75	4.63	6.50	7.00
Na-desoxycholate (+4 °C 16 hrs)	4.13	3.25	1.50	0	2.87	2.75	4.25	0	6.00
Control	5.75	6.13	4.83	4.75	4.75	4.50	4.75	6.50	7.00
Saponin (+4 °C 16 hrs)	5.50	4.63	4.08	3.75	3.75	3.50	4.50	2.50	6.00

* Infective titre in $\log_{10}$ $TCID_{50}$/0.1 ml; ** Porcine enterovirus.

In the 0.1% solution of saponin, the "CKp" strain and the virulent strain FC lost infectivity by 0.25, the attenuated strain Hannover by 0.75, the attenuated Purdue and the virulent BB and ESZT strains by 1.00, whereas the attenuated strain FS 216/64 lost infectivity by 1.50 $\log_{10}$ $TCID_{50}$ (Table I).

Results of trypsin-sensitivity tests are shown on Fig. 1. Treatment with 0.5% trypsin at 37 °C reduced the original titre of the "CKp" strain by

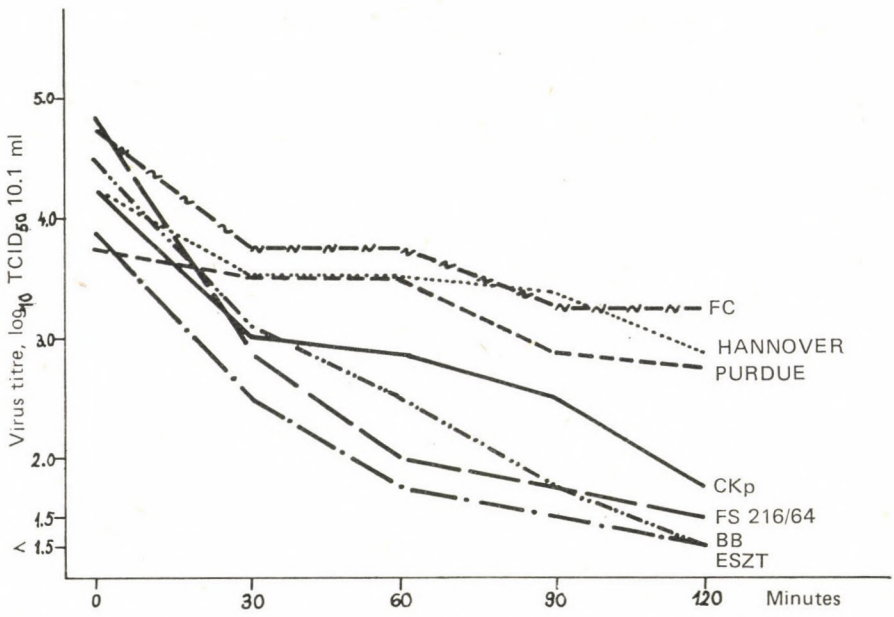

Fig. 1. Sensitivity of different TGEV strains to 0.5% trypsin at 37 °C

Table II

Effect of 2% polyethylene glycol (PEG) on the plaque production of different TGEV strains

TGEV strains	Plaque formation inhibition (%)
CKp	18
FS 216/64	39
Hannover	53
Purdue	33
BB	27
ESZT	33
FC	28

2.50 $\log_{10}$ $TCID_{50}$ in 120 min. The infectivity of the attenuated strain FS 216/64 decreased by 3.37 $\log_{10}$ $TCID_{50}$, while the virulent strains BB and ESZT were completely inactivated in 120 min. The attenuated strains Purdue and Hannover, as well as the virulent strain FC were more resistant. The respective titre losses were 1.00, 1.38 and 1.50 $\log_{10}$ $TCID_{50}$.

The effect of PEG on the plaque production is shown in Table II. Briefly, PEG in a final concentration of 2% exerted a less than 50% reduction in the plaque production of all the TGEV strains tested, except the attenuated

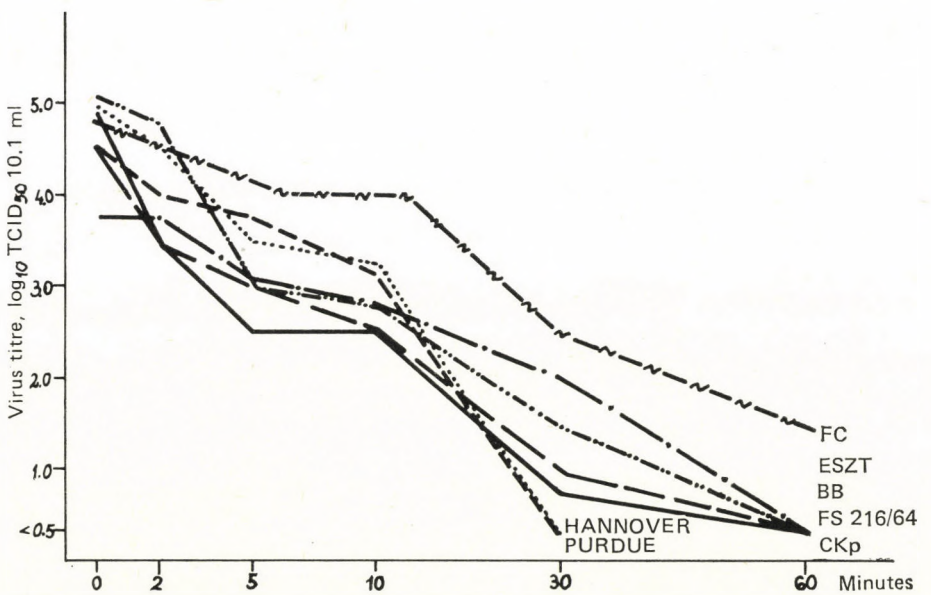

Fig. 2. Thermal sensitivity of different TGEV strains at 50 °C

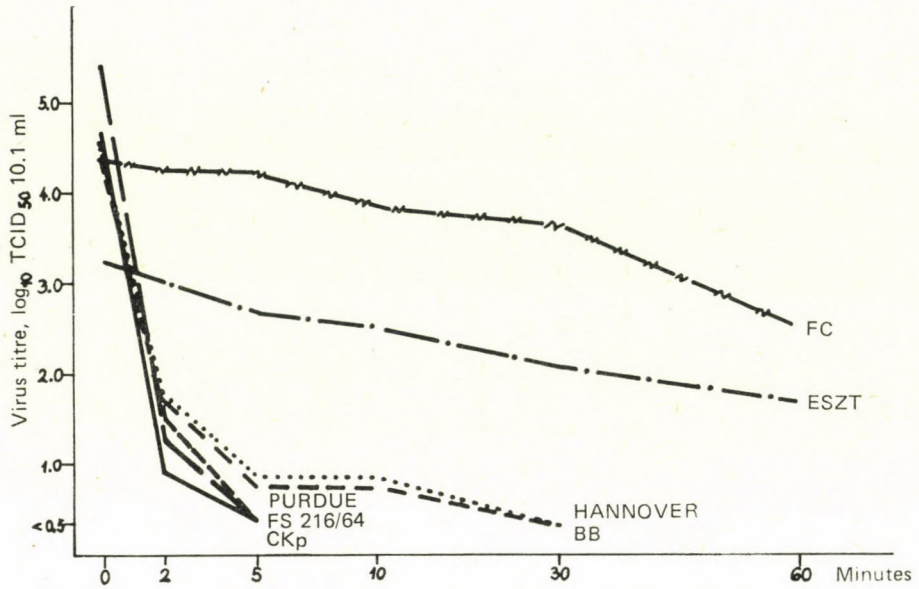

Fig. 3. Sensitivity of different TGEV strains to 50 °C in the presence of 1 *M* $MgCl_2$

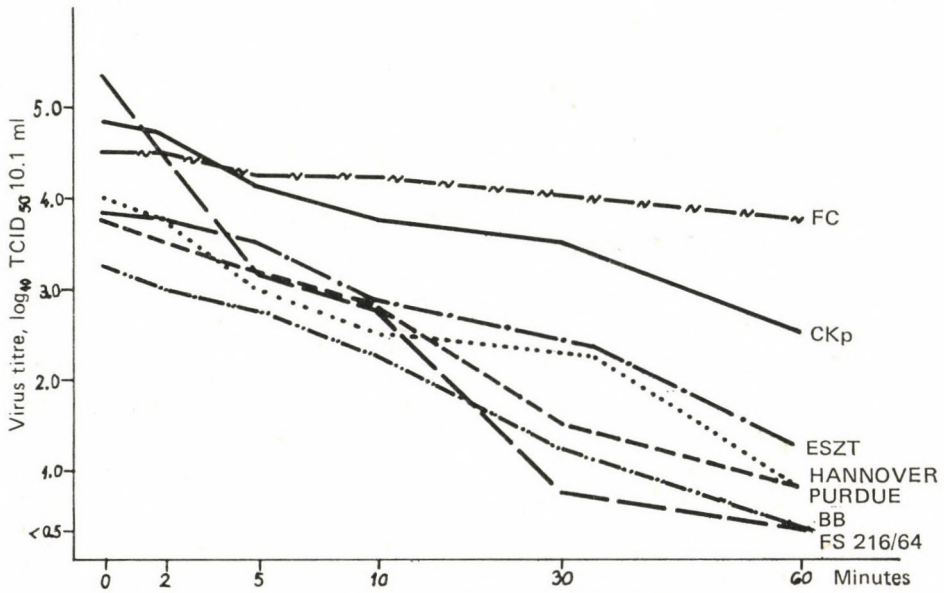

Fig. 4. Sensitivity of different TGEV strains to 50 °C in the presence of 1 *M* $MgSO_4$

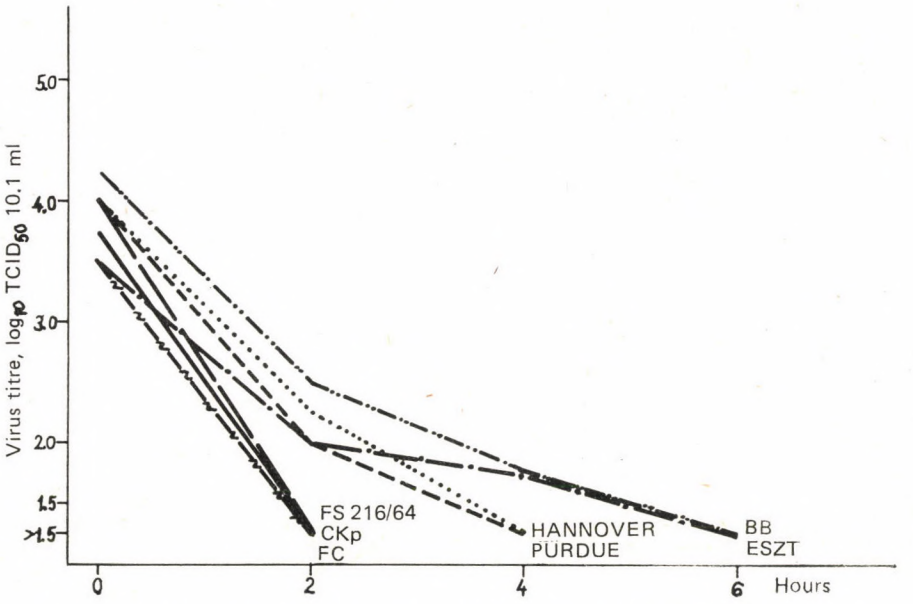

Fig. 5. Sensitivity of different TGEV strains to pH 3.0 at 37 °C

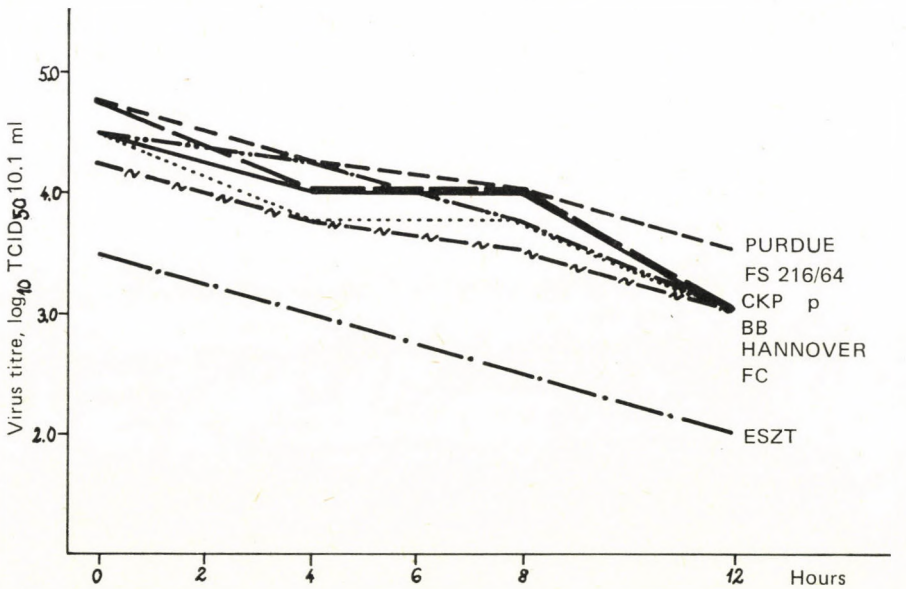

Fig. 6. Sensitivity of different TGEV strains to pH 6.8 at 37 °C

TGEV strain Hannover. The plaque production of this latter TGEV strain was reduced by 53%.

Dynamics of the thermal inactivation of different TGEV strains at 50 °C is demonstrated in Fig. 2. The infectivity of the "CKp" TGEV strain and the virulent strains BB and ESZT, as well as that of the attenuated strain FS 216/64 was completely destroyed when held at 50 °C for 60 min. Two attenuated strains Hannover and Purdue lost their infectivity within 30 min. The virulent strain FC proved to be more stable at this temperature. Although, a constant 3.25 $\log_{10}$ of titre loss was recorded after 60 min, a 1.50 $\log_{10}$ $TCID_{50}$ of virus infectivity remained.

The sensitivity of different TGEV strains to 50 °C in the presence of 1 *M* $MgCl_2$ is compared on Fig. 3. The "CKp" vaccine strain and the attenuated strains FS 216/64 and Purdue lost their infectivity within 5 min, while the attenuated Hannover and the virulent BB strains were inactivated in 30 min. Two virulent strains, ESZT and FC, showed a moderate loss of titre after a 60-min exposure (1.58 and 1.88 $\log_{10}$ $TCID_{50}$, respectively).

In the presence of 1 *M* $MgSO_4$, the thermal sensitivity of TGEV strains is shown on Fig. 4. The infectivity of the "CKp" strain decreased by 2.33 $\log_{10}$ $TCID_{50}$ after 60 min. The virulent ESZT and the attenuated Hannover and Purdue strains showed a similar (2.00 to 3.75 $\log_{10}$ $TCID_{50}$) reduction of titre, whereas the infectivity of both the attenuated strain FS 216/64 and the virulent strain BB was destroyed within 60 min. No significant loss of infectivity was recorded with the virulent strain FC in the presence of 1 *M* $MgSO_4$. Its infectivity decreased by 0.75 $\log_{10}$ $TCID_{50}$ during a 60-min incubation at 50 °C.

At pH 3 (Fig. 5), the infectivity of the "CKp" strain was destroyed within 2 hrs at 37 °C. Similar results were obtained with the attenuated strain FS 216/64 and with the virulent strain FC. The infectivity of the attenuated strains Hannover and Purdue was inactivated in 4 hrs, whereas the virulent strains BB and ESZT lost their infectivity in 6 hrs. At pH 6.8, both the virulent and the attenuated TGEV strains showed an approximately 1.5 $\log_{10}$ $TCID_{50}$ loss of infectivity in 12 hrs at 37 °C (Fig. 6).

Discussion

No substantial differences were found between field and cell culture-attenuated TGEV strains in the chloroform, sodium deoxycholate and saponin sensitivity tests. All the strains were sensitive to the treatment with chloroform and 0.1% sodium deoxycholate and — except the attenuated strain Purdue — proved to be more sensitive to the action of 0.1% sodium deoxycholate at 37 °C than at +4 °C. This latter strain showed an elevated sensitivity to the action of this detergent both at +4 °C and at 37 °C. All the TGEV strains showed a moderate sensitivity to the action of 0.1% saponin.

Likewise, no significant differences could be demonstrated between field and cell culture-attenuated TGEV strains in the thermal inactivation at 50 °C and stability at pH 3. Generally, the thermal inactivation of different TGEV strains was enhanced by 1 *M* $MgCl_2$. The thermal resistance of most of the TGEV strains tested was slightly enhanced by 1 *M* $MgSO_4$. One possible explanation for this phenomenon might be the different sensitivity of TGEV strains to SO^{--} and Cl^- ions.

The marked differences found among various TGEV strains in the sensitivity to 0.5% trypsin could neither be linked with their virulence. Four strains, among them the "CKp" vaccine strain and two virulent strains of low cell culture passage level, proved to be more sensitive to the action of trypsin, while the virulent FC and other two cell culture-attenuated TGEV strains showed a lower sensitivity to this proteolytic enzyme.

Furuuchi et al. (1975) also found marked differences among TGEV strains in the sensitivity to proteolytic enzymes and they established a significant correlation between the pathogenicity of strains and their *in vitro* sensitivity to digestive enzymes. In contrast to these findings, the present study revealed no substantial differences between field and cell culture-attenuated TGEV strains based on their physicochemical properties. The variation in stability to different physicochemical treatments and in the resistance to proteolytic enzyme appears to be a specific property of different TGEV strains and does not seem to be influenced by the cell-culture passage number and by the level of the attenuation.

Summary

Selected physicochemical properties of three field and four cell culture-attenuated strains of TGEV were compared. All the strains were sensitive to the treatment with chloroform and 0.1% sodium deoxycholate, and showed a moderate sensitivity to the action of 0.1% saponin. No significant differences could be demonstrated between field and cell culture-attenuated strains in the thermal inactivation at 50 °C and stability to pH 3. 1 *M* $MgCl_2$ generally enhanced the thermal inactivation of the TGEV strains, while 1 *M* $MgSO_4$ slightly promoted their thermoresistance. The marked differences found among TGEV strains in the resistance to the action of 0.5% trypsin did not correlate with their virulence.

It is concluded that the variation in stability to different physicochemical treatments and in resistance to proteolytic enzyme is a specific property of different TGEV strains and does not seem to be influenced by either the cell culture passage number or the level of the attenuation.

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STUDIES ON THE EGG-DROP SYNDROME

By

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Drop of egg production accompanied by defective shell calcification has occurred recently in large parent and laying flocks in several countries (Berry, 1969; Eck et al., 1976; McFerran et al., 1972; McFerran and Adair, 1977; McFerran and Connor, 1977; McFerran et al., 1978). In the reported cases, metabolic diseases, Newcastle disease, infectious bronchitis and any other known infectious disease of chicken could be ruled out (Crinion et al., 1971). Besides various types of adenovirus, an adenovirus-like haemagglutinating agent unrelated to any known adenovirus serotype was isolated in certain affected flocks (McFerran et al., 1978).

In the autumn, 1977, we observed drop of egg production connected with production of abnormal eggs in a diseased large layer flock. Closer examinations made on several birds before stamping out of the flock are reported in this paper.

Materials and methods

Experimental animals fifteen layers were killed by exsanguination in different stages of the disease for pathological, light microscopic, electron microscopic, bacteriological, virological and serological examination and blood analysis.

Light microscopy. Specimens were taken from spleen, liver, kidney, small and large intestine and pancreas; the pituitary, adrenal and thyroid glands, ovary and oviduct were removed and processed *in toto*. The samples were fixed in 10% formalin and embedded in paraffin. The oviduct was processed with the so-called "scroll-technique" proposed by Széky et al. (1977). The paraffin sections were stained with Mayer's acid haemalaun-eosin for general orientation, frozen sections with Sudan III. The technique proposed by Tanka—Keller (1974) was employed to distinguish adenohypophyseal acidophilic and basophilic cells.

Electron microscopy. The haemagglutinating BC 14 antigen (Intervet Laboratories Ltd.) was used as reference standard, and the centrifugal supernatant (5000 rpm., 20 min) of intestinal content from diseased birds as test material. Antigen and test material were both added to 0.01% bovine serum albumin solution by drops until a slight opalescence appeared. To this mixture an equal amount of convalescent chicken serum was added to produce aggluti-

nation, and finally 3% phosphotungstic acid (PTA) solution was added as counter-stain. One drop of the suspension thus obtained was mounted on a grid coated with Formwar membrane, dried, and observed in a Philips 201 CS electron microscope.

Serological tests. Takátsy's micro technique (TAKÁTSY, 1955) was used throughout. The haemagglutination-inhibiting serum antibodies present in paired serum samples taken in the initial stage of the disease and 2—3 weeks later were titrated against 8 haemagglutinating (HA) units of the BC 14 adenovirus antigen.

Pathological and microbiological testing

The pathological and bacteriological examinations, as well as the virological examinations for Newcastle disease and infectious avian bronchitis virus, were made by the diagnostic procedures routinely employed in this Institute.

Chemical tests

These included determination of blood calcium and standard bicarbonate levels, and haematocrit value.

Results

The symptoms of the disease started between the 3rd and 7th weeks of laying. Some 10% of the birds developed watery diarrhoea, which was followed after a few days by decrease of laying and production first of soft-shelled eggs, later of ones with abnormally calcified shell. The feed consumption of the birds remained unchanged, and the mortality was negligible. The outbreak lasted 2—4 weeks within a group or house. The symptoms were irresponsive to dietary measures including extra calcium supply. The egg production curve is shown in Fig. 1.

Individual examinations of birds also indicated that the disease started with diarrhoea. Initially, the faeces was of greenish colour and contained blood streaks, later it became watery, either colourless or greyish-white owing to presence of uric acid and was discharged practically without interruption. At this stage, most layers still produced normal eggs, but egg retention also occurred in some cases. The watery diarrhoea subsided gradually in 5—9 days, but then the birds began to lay abnormal eggs.

The first sign of abnormality was the production of soft-shelled eggs practically devoid of a hard shell (Figs 2 and 3). Later, shell calcification was

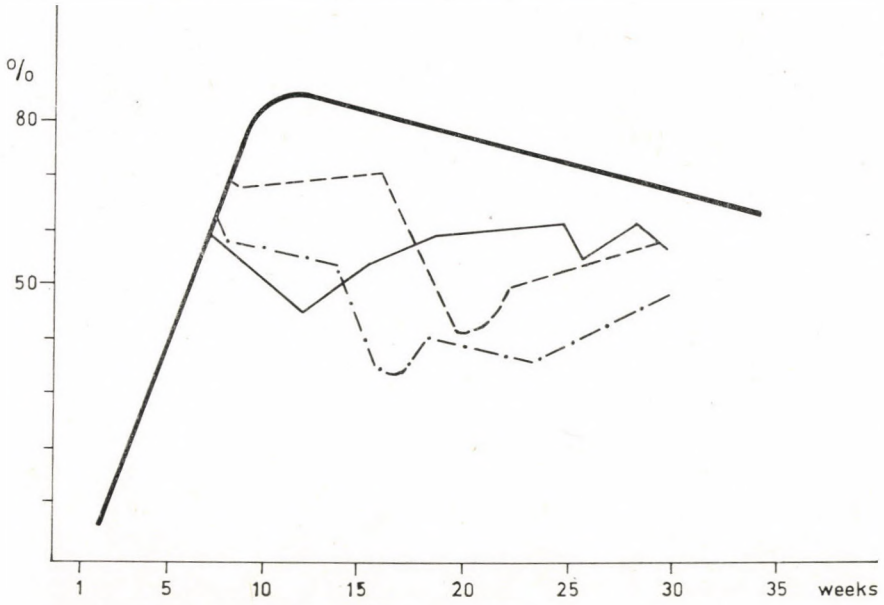

Fig. 1. The egg production curve in the affected groups; ——— normal; ———, -·-·-·-·-, – – – – – infected

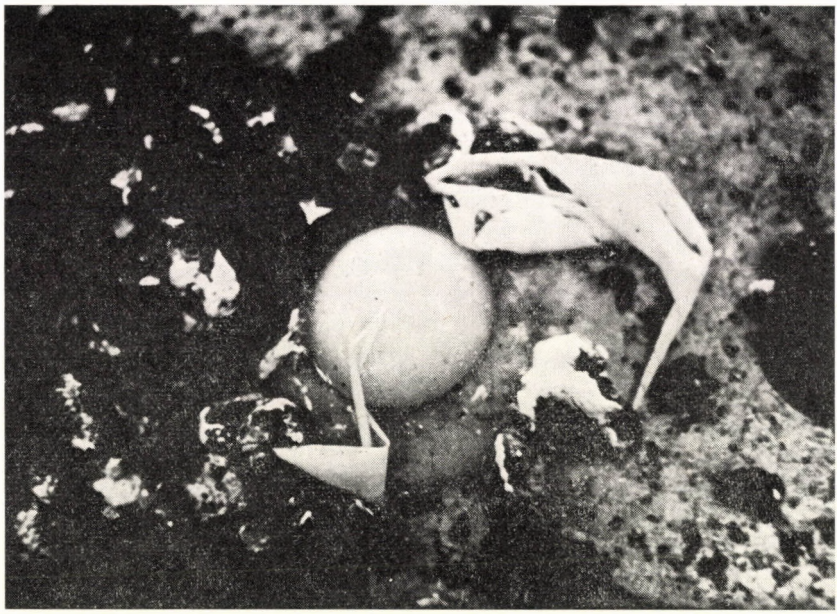

Fig. 2. Soft, so-called skin-shelled, impaired egg in the litter. The inner structure of the egg was intact

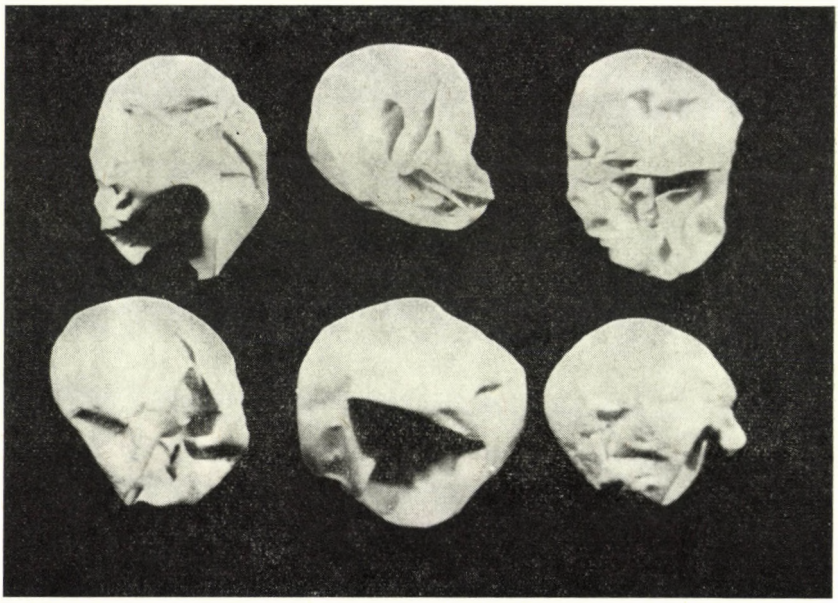

Fig. 3. Soft, so-called skin-shelled eggs, partially dehydrated at the time of collection

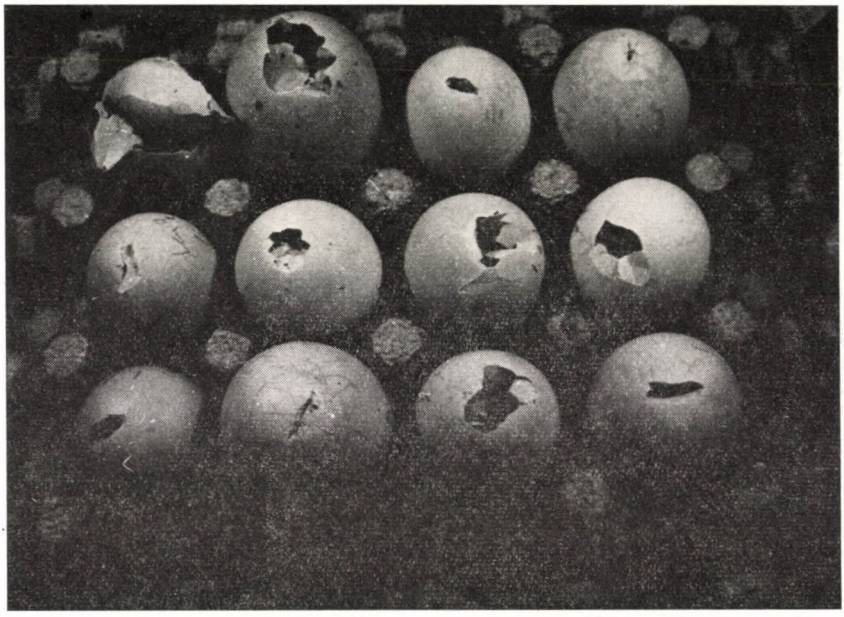

Fig. 4. Abnormally pigmented, thin-shelled, cracked eggs produced in the final stage of disease after a protracted course

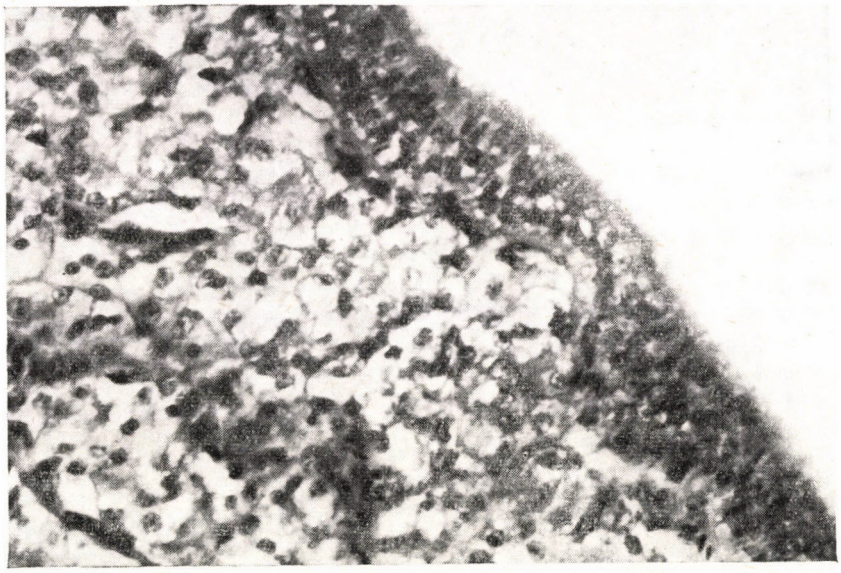

Fig. 5. Detail of the oviduct from a healthy layer producing normal eggs. H.E., ×400

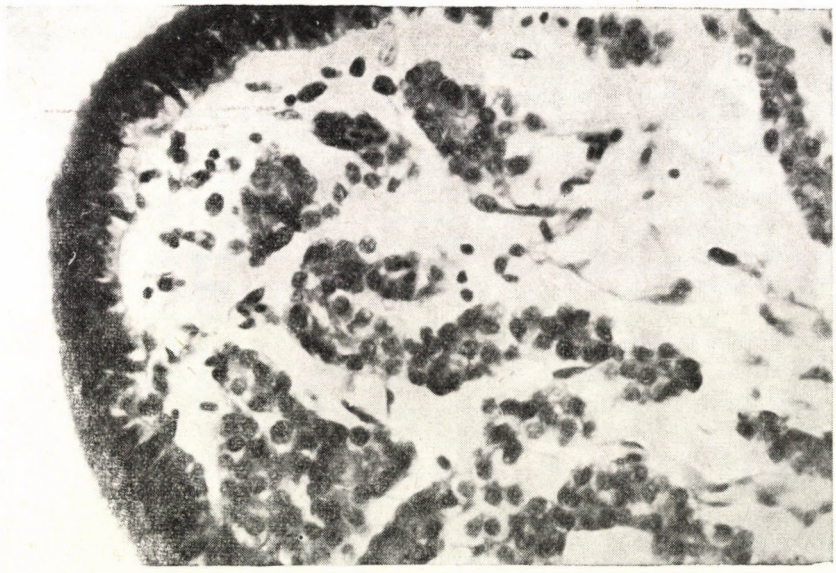

Fig. 6. Detail of the oviduct from an affected layer at the stage of soft-shelled egg production. Note oedema and drawing apart of the calciferous glands in the uterine mucosal folds. H.E., ×400

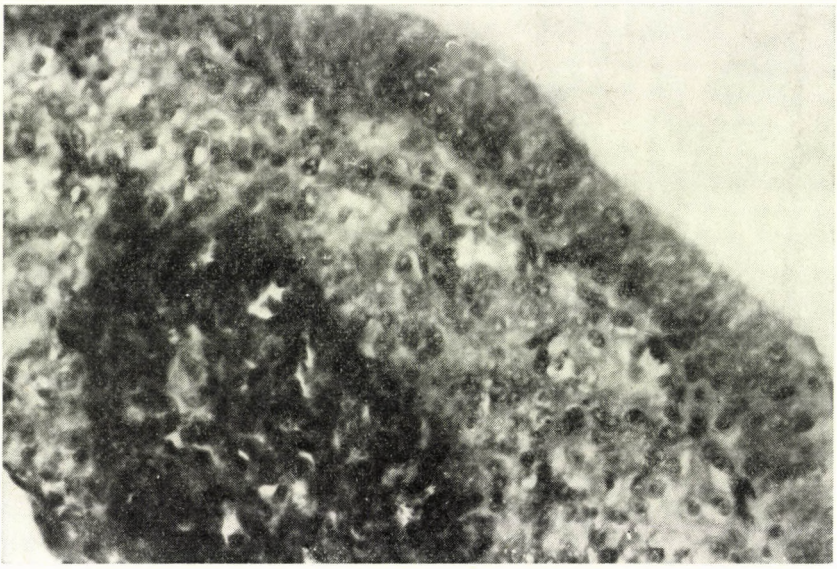

Fig. 7. Detail of the oviduct from a diseased layer at the stage of defective egg-shell calcification. Note perivascular lymphoid hyperplasia in a uterine mucosal fold. H.E., ×400

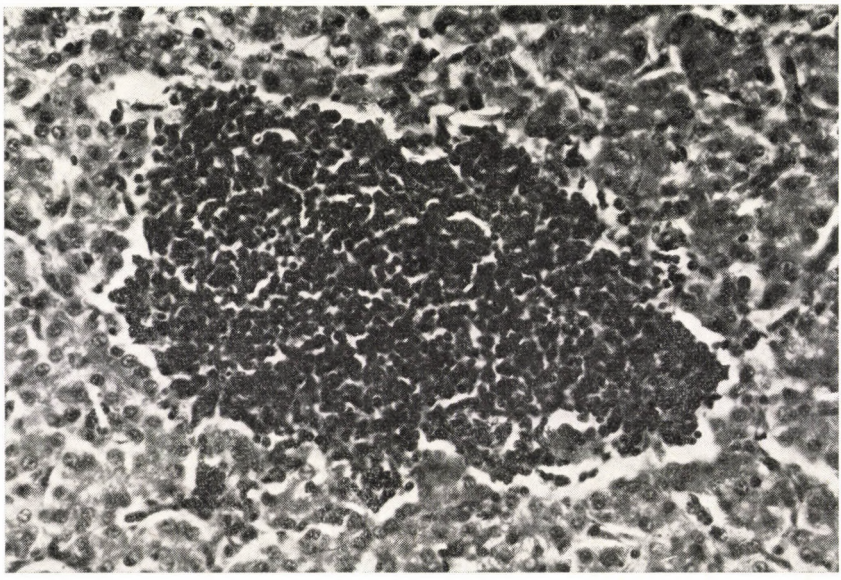

Fig. 8. Detail of the liver from a diseased layer at the stage of defective egg-shell calcification. Note hyperplastic lymph follicle. H.E., ×240

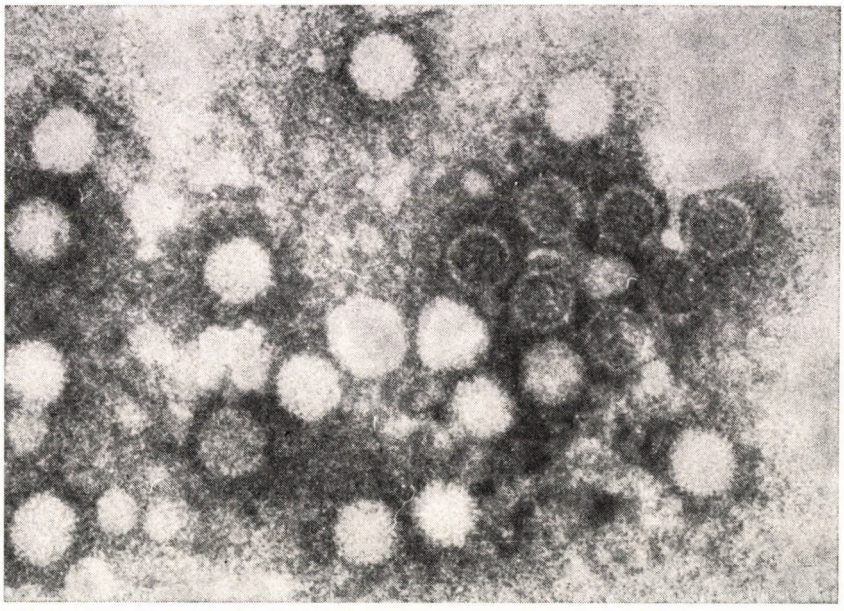

Fig. 9. Electron micrograph of agglutination of BC 14 antigen by convalescent chicken serum. Negative staining, ×144 800; inset at top right, ×210 000

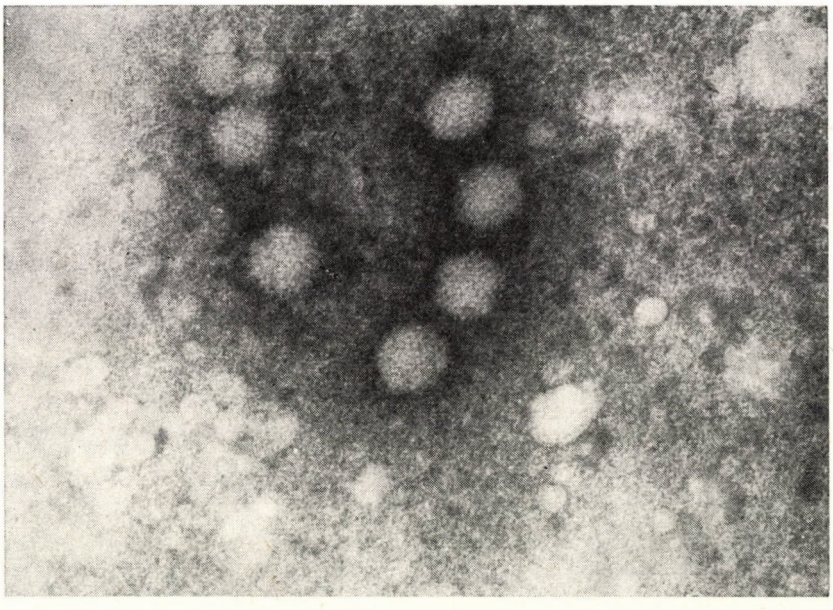

Fig. 10. Electron micrograph of agglutination of virus-like particles present in the intestinal contents of acutely diseased birds by convalescent serum. Negative staining, ×144 800; inset at top right, ×210 000

gradually restored, but the eggs laid in the terminal stage of the disease still had extremely thin, poorly pigmented and very fragile shells; many eggs cracked spontaneously when touched the laying cage (Fig. 4). The illness generally lasted 2—6 weeks; its course was sometimes interrupted by temporary improvement, followed by return of the symptoms. None of the birds observed individually died of the disease.

Gross lesions related to the condition were not found at any stage of the disease. No ossification disorders could be detected either: the bone strength was found normal at the given age.

Light microscopic examination revealed characteristic changes in the oviduct wall of birds killed at the stage of soft-shelled egg production. The mucosal propria of the uterus was oedematous, in a rising degree towards the ridges of the mucosal folds, causing these to round off or, occasionally, to take a club-like shape. The oedema pushed apart the outlets of the calciferous glands, thus giving rise to a marked looseness of the glandular tissue and a slight atrophy of the gland outlets (Fig. 5) compared to normal (Fig. 6). A slight mononuclear cell infiltration was also seen in the oedematous tissue. At the stage of improving shell calcification, the oedema of the uterine mucosa became less marked, but focal or diffuse infiltration by mononuclear cells was still found. Formation of lymphoid follicles took place along the walls of the major blood vessels (Fig. 7). The same lesions appeared in a much milder from in other parts of the oviduct.

In nearly every case, lymphoid follicles also developed in the liver (Fig. 8) and kidneys. Birds killed during the diarrhoeic stage showed an acute serous inflammation of the mucosal lining in the mid- and lower intestine. After the cessation of diarrhoea, the enteritis took a subacute course and was accompanied by lymphoid tissue hyperplasia. The CNS and the endocrine system showed no microscopic lesion attributable to the disease.

Electron microscopically virus-like particles of icosahedral symmetry, 65—70 nm in diameter, corresponding in shape and size to the virions present in the BC 14 adenovirus antigen, were detected in the intestinal contents of diarrhoeic layers. The particles were agglutinated by the convalescent sera in the same titre as the reference antigen (Figs 9 and 10).

The HI test indicated rising antibody levels in the sera taken from birds in the initial stage of the disease and in groups of birds sampled before the onset of the disease and in the convalescence.

We failed to isolate bacteria, NDV or avian bronchitis virus from different organs, including the reproductive organs.

The serum calcium and standard bicarbonate levels, and the blood haematocrit value were within the physiological range throughout.

Discussion

The symptoms had initially aroused the suspicion of a metabolic disease. The same was suggested by the unchanged mortality despite the high morbidity rate, absence of gross lesions and lack of pathogenic bacteria, NDV and avian bronchitis virus. However, the course of the disease was not affected by dietary measures such as *ad libitum* calcium and elevated vitamin supplements.

The microscopic lesions found in the uterus corresponded to those observed by ECK et al. (1978) and suggested the involvement of an infectious agent.

Electron microscopic examination of the intestinal contents from layers killed during the acute diarrhoeic stage of the disease, showed virus-like particles in every respect similar to the virions present in the BC 14 adenovirus antigen preparation (MADELEY, 1972). The electron microscopic finding and the serological evidence of rising antibody levels to the BC 14 antigen have affirmed the aetiological importance of the agent, and support the conclusion that the haemagglutinating adenovirus, also demonstrated in the Netherlands and Northern Ireland, plays a role in the so-called egg-drop syndrome.

Further information emerging from our studies is that the microscopic lesions appearing in the oviduct during the acute stage were caused by the infectious agent. The inflammation of the uterus can in itself account for the abnormal shell calcification (STURKIE, 1976). The presence of hepatic and renal lymphoid hyperplasia and follicle formation during the advanced stage of the disease indicates that the agent detected in the intestinal tract also affects organs other than reproductory if the disease takes a protracted course.

Summary

A disease accompanied by drop of egg production and abnormal shell calcification was observed in a large layer flock in 1977. The disease started with diarrhoea which soon became watery and was followed by reduction of the egg production and production of abnormal eggs with soft, defectively calcified, deformed or extremely thin shell. No increase of losses occurred during the mass incidence of the disease, nor was there a reduction in feed consumption. The condition lasted 2—4 weeks, followed in certain cases by recurrent fluctuations of the egg production. Birds killed by exsanguination at different stages of the disease showed no gross lesions, but histological examination revealed an inflammatory involvement of the oviduct, above all of the uterine mucosa, which initially showed oedema and degeneration of the calciferous glands, later mononuclear cell infiltration and lymphoid hyperplasia. The latter change also appeared in other organs in the advanced stage of the disease. Immuno-electron microscopic examination of samples taken from intestinal contents during the acute diarrhoeic stage, virus-like particles in every respect similar to those present in the adenovirus antigen preparation BC 14 were detected. In paired serum samples taken in the initial stage of the disease and 2—3 weeks later rise of antibody titres to the BC 14 antigen was demonstrated.

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EFFECT OF IRRADIATION ON THE VIABILITY AND TOXIN PRODUCTION OF DIFFERENT FUNGUS SPECIES

By

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Feeding stuffs are commonly heat-treated before storage, although this procedure cannot eliminate all risks of contamination. Heat exposure kills the *Fusarium* fungi, but does not affect their toxic products, and many grains split by heat present themselves later as easily accessible growth media to storehouse moulds.

Among the sterilization-preservation procedures employed in the food industry, radiation treatment has been recognized as the method of choice for satisfying current demands. In view of this, it seemed worthwhile to investigate whether radiation sterilization could prevent mould growth and mycotoxin production, and inactivate the toxin(s) already present in the stored feeding stuffs.

The problem has been little studied, and conflicting opinions have been reported. APPLEGATE and CHIPLEY (1973, 1974) found that exposure to less than 200 Krad doses of gamma irradiation enhanced rather than depressed aflatoxin and ochratoxin production by *Aspergillus flavus* and *A. ochraceus*, respectively. Against this, BULLERMAN and HARTUNG (1974) observed depression of spore and, especially, mycelium development by low doses of gamma irradiation. Two strains (an *A. flavus* and an *A. parasiticus)* responded differently to irradiation with 100 krad, by increase and decrease of later toxin production, respectively, but uniformly to 200 krad, which depressed the toxin production of both.

Exposure to 100 krad depressed the growth rate of *Penicillium patulum* to 81%, and its toxin production to 64%, relative to the untreated control. The respective values for 200 krad irradiated cultures were 40 and 1%, (BULLERMAN and HARTUNG, 1975). The effects of the same treatment on other strains followed similar tendencies.

In this laboratory, preliminary investigations into the problem had been begun in 1973. The radiation dose required to kill pure cultures of *Fusarium graminearum* had then been established as 1 Mrad.

Further preliminary studies performed in 1974 and 1975 revealed that exposure in the dose range of 0.2—0.5 Mrad failed to arrest the growth of fusaria, and even seemed to stimulate toxin production at a certain dose performance. Elevation of the dose accounted for retardation of mould growth, but the quantity of the toxic product tended to increase as long as living fungal cells were present. Feeding experiments performed in the pig production unit of the State Farm Baja indicated that F-2-containing corn treated with 2 Mrad still was biologically active.

Further studies conducted during the last years were centered on the establishment of those irradiation parameters which might effectively prevent either the growth or the toxin production of toxic *Fusarium*, *Aspergillus* and *Penicillium* strains isolated from feeding stuffs used in Hungary.

Irradiation was carried out by the Radiation Work Group of the PHYLAXIA Veterinary Biologicals and Feedstuffs Co. Ltd. Budapest.

Materials and methods

Experiments with Fusarium culmorum

The *Fusarium culmorum* strain F79 of our strain collection, noted for intensive toxin production, was used. 4400 g autoclaved corn of good quality was inoculated with a malt broth culture of the strain. Inoculated samples were thoroughly shaken during the initial days of culturing to ensure even distribution of the fungus cells on the kernels before the development of a dense mycelial mass. The infected corn was incubated for 14 days, which were sufficient for abundant growth of the fungus throughout the kernel mass. The kernel culture was divided into three parts after incubation, and one part was heated in an exsiccator for 6 hrs at 80 °C.

From the heat-treated corn, 88 samples, each sealed in a bag, were prepared in the PHYLAXIA institute. Sixty-two samples were exposed to ionizing gamma irradiation of various parameters (Table I).

After incubation for different times and at different temperatures, as shown in Table I, 10 kernels were collected from each sample for re-isolation of *F. culmorum*. The kernels were washed first in 0.1 N NaOH, then in sterile distilled water, and were halved under sterile conditions. The halved kernels were placed on Sabouraud agar. After incubation for 6 days at 28 °C, the Fusarium colonies were counted on plate.

Table I

Scheme of the experiment

Time of treatment	Control	No. of samples		Heated + irradiated
		heated	irradiated	
After initial growth phase (at 24—28 °C)	1	1	—	—
After 2-week cold exposure (12—14 °C)	1	1	9	3
After 4-week cold exposure (12—14 °C)	1	1	9	3
After re-exposure to growth temperature (24—28 °C)	1	1	9	3
	4	4	27	9=44

On a total 88 samples were tested in two replica series.
Irradiation: dose 0.5, 1.0 or 1.5 Mrad,
dose performance 20, 250 or 500 krad/h.
Combined treatment: exposure of heated samples to the above doses at 250 Krad/h dose performance.

The remaining samples were dried to constant weight, and the parallel samples were mixed pooled to minimize the technical error resulting from weight loss. Thus the original number of samples was reduced to one half, from 88 to 44.

F-2 toxin was determined by the semi-quantitative chromatographic procedure proposed by VÁNYI et al. (1973). The chromatograms were evaluated by comparison of the fluorescent spots to an appropriate reference standard. For complementary evaluation, we used the colour reaction proposed by SARUDI (1974).

Each sample was chromatographed in two parallel series; thus, by evaluation in UV and visible light, four values were obtained for each.

Experiments with Aspergillus ochraceus *and* Penicillium urticae

The ochratoxin-A-producing *Aspergillus ochraceus* strain 1537/2 and the patulin-producing *Penicillium urticae* strain 51 of our collection were used. The cultures were grown on malt agar, which promotes abundant spore formation.

After culturing for 4 days, 2 loopfuls of spores were collected from each culture and were suspended in 10 ml sterile saline (0.7% NaCl), containing one drop of detergent Tip 67.

The stock suspensions were diluted in tenfold serial steps (range: 10^{-2}—10^{-6}), and 1 ml of each dilution was used for pour-plates of Sabouraud agar, to determine the initial spore count. One replica and one control series was set up with each test.

Another 1-ml sample of each dilution was pipetted into an agglutination tube, sealed with a rubber stopper, and exposed to irradiation at three different levels of dose and dose performance (0.5, 1.0, 1.5 Mrad; 250, 400, 500 krad/hr). Including the parallel series, on a total 36 samples were irradiated.

The irradiated samples were then diluted and plated as above, and incubated for 72 hrs at 26 °C, for re-isolation and colony counting.

The radiation-resistant isolates were then examined for toxin production. Four colonies with about 1 cm^2 surface area were removed from the Sabouraud agar with a hot loop, and were transferred to the surface of a nutrient broth containing yeast extract and sucrose. During incubation for 6 days at 26 °C, the vigorously growing fungi formed a film over the entire surface of the growth medium. The fungal toxin was extracted from the outgrowth (ROMVÁRY and SELLYEY, 1976), and was layer-chromatographed for semi-quantitative determination.

Results

Experiments with Fusarium culmorum

The re-isolation data of *F. culmorum* are shown in Table II. Among the 88 samples, 28 were contaminated by other fungi. The F-2 contents of the samples are shown in Table III and Fig. 1. All values represent means for parallel assays.

Experiments with Aspergillus ochraceus *and* Penicillium urticae

The pre- and post-irradiation spore counts of the *A. ochraceus* cultures are shown in Table IV. As in this instance the irradiation seemed 100% effective the experiment was repeated to check the validity of this finding. The second experiment substantiated the 100% fungicidal effect of gamma irradiation on *A. ochraceus*.

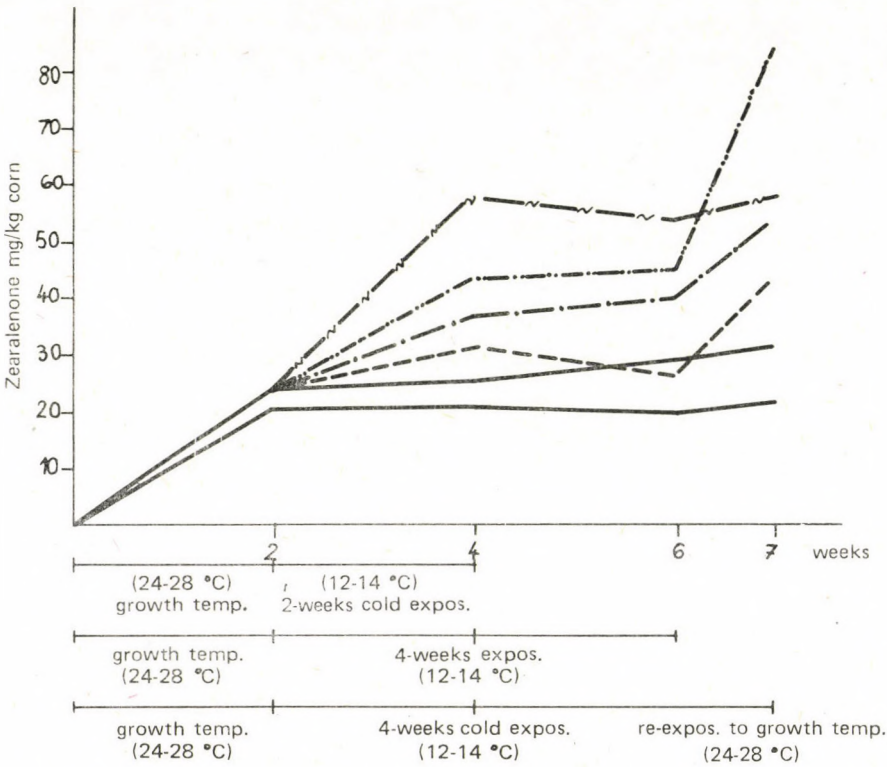

Fig. 1. Influence of heating and irradiation on F-2 toxin production. —··— irradiated, Dp: 500 krad/h, D: 1.0 Mrad; –∼–∼– heated and irradiated; —·— irradiated, Dp: 250 krad/h, D: 1.0 Mrad; – – – – irradiated, Dp: 20 krad/h, D: 1.0 Mrad; ——— control, heated control

Table II

Re-isolation of *Fusarium culmorum* from experimentally infected corn samples after different treatments (from 10 kernels per sample)

	After initial growth phase (at 24—28 °C)		After two-week cold exposure		After four-week cold exposure		After re-exposure to growth temperature	
Control	8	10	9	8	10	$9^{(P)}$	10	8
Heated control	2	0	$2^{(P)}$	$0^{(P)}$	$0^{(P)}$	$0^{(P)}$	$0^{(P)}$	$0^{(P)}$
Irradiated samples:								
Dp: 20 krad/h D: 0.5 Mrad	—	—	10	7	10	10	10	9
Dp: 20 krad/h D: 1.0 Mrad	—	—	7	4	10	10	10	10
Dp: 20 krad/h D: 1.5 Mrad	—	—	$8^{(A)}$	5	10	10	9	4
Dp: 250 krad/h D: 0.5 Mrad	—	—	7	$4^{(Al)}$	10	10	10	$8^{(P)}$
Dp: 250 krad/h D: 1.0 Mrad	—	—	9	$8^{(P)}$	10	10	10	10
Dp: 250 krad/h D: 1.5 Mrad	—	—	9	6	10	9	10	$8^{(H)}$
Dp: 500 krad/h D: 0.5 Mrad	—	—	10	10	10	8	10	9
Dp: 500 krad/h D: 1.0 Mrad	—	—	9	$7^{(A+P)}$	10	10	10	$9^{(Y)}$
Dp: 500 krad/h D: 1.5 Mrad	—	—	10	8	10	6	8	5
Combined treatment (heating + irradiation)								
Dp: 250 krad/h D: 0.5 Mrad	—	—	$0^{(H)}$	$0^{(H+P)}$	$0^{(P)}$	$0^{(P)}$	$0^{(P)}$	$0^{(P)}$
D: 1.0 Mrad	—	—	$0^{(H)}$	$0^{(Y)}$	$1^{(A)}$	$0^{(P+H)}$	5	2
D: 1.5 Mrad	—	—	1	0	$1^{(Al+P+A+Y)}$	$0^{(P+H+Al)}$	$0^{(H)}$	$0^{(Al)}$

Dp, dose performance; D, dose; Other fungal contamination: P, *Penicillium*; R, *Rhisopus*; A, *Aspergillus*; Al, *Alternaria*;, H, *Hormodendrum*; Y, yeasts.

Table III

F-2 content of the samples (ppm)

	After initial growth phase (at 24—28 °C)	After two-week cold exposure	After four-week cold exposure	After re-exposure to growth temperature
Control	23.1	24.5	28.5	31.2
Heated control	20.8	20.4	19.3	21.5
Irradiated samples:				
Dp: 20 krad/h D: 0.5 Mrad	—	22.3	38.2	47.6
Dp: 20 krad/h D: 1.0 Mrad	—	32.2	26.2	43.0
Dp: 20 krad/h D: 1.5 Mrad	—	21.5	45.0	37.5
Dp: 250 krad/h D: 0.5 Mrad	—	33.1	20.2	66.8
Dp: 250 krad/h D: 1.0 Mrad	—	36.0	39.1	53.2
Dp: 250 krad/h D: 1.5 Mrad	—	16.5	18.0	38.5
Dp: 500 krad/h D: 0.5 Mrad	—	25.4	62.5	83.7
Dp: 500 krad/h D: 1.0 Mrad	—	42.6	44.3	83.8
Dp: 500 krad/h D: 1.5 Mrad	—	22.4	34.2	33.4
Combined treatment:				
Dp: 250 krad/h D: 0.5 Mrad	—	56.6	41.2	53.7
Dp: 250 krad/h D: 1.0 Mrad	—	55.1	52.6	57.8
Dp: 250 krad/h D: 1.5 Mrad	—	40.0	57.3	58.6

Table IV

Spore counts (no./ml) in samples infected with *Aspergillus ochraceus*

	I	II
Before irradiation	4.200 000	3 200 000
After irradiation		
Dose: 0.5 Mrad		
Dose performance: 250 krad/h	—	—
400 krad/h	—	—
500 krad/h	—	—
Dose: 1.0 Mrad		
Dose performance: 250 krad/h	—	—
400 krad/h	—	—
500 krad/h	—	20 000
Dose: 1.5 Mrad		
Dose performance: 250 krad/h	—	—
400 krad/h	—	—
500 krad/h	—	—

Table V

Spore counts (No./ml) in samples infected with *Penicillium urticae*

	I	II
Before irradiation	6 300 000	5 100 000
After irradiation		
Dose: 0.5 Mrad		
Dose performance: 250 krad/h	20 000	100 000
400 krad/h	1 000	20 000
500 krad/h	—	1 000
Dose: 1.0 Mrad		
Dose performance: 250 krad/h	20 000	10 000
400 krad/h	10 000	1 000
500 krad/h	—	—
Dose: 1.5 Mrad		
Dose performance: 250 krad/h	1 000	10 000
400 krad/h	2 000	1 000
500 krad/h	—	—

The spore counts per ml assessed in *Penicillium urticae* cultures are shown in Table V. The quantities of patulin and ochratoxin produced by the radiation-resistant cultures in synthetic medium are shown in Table VI. (Visual evaluation was based on the comparison of the size of fluorescent and non-fluorescent mycotoxin spots.)

Discussion

Fusarium culmorum was re-isolated from the control samples, and the re-isolate produced the expected quantity of toxin at appropriate temperatures.

From the heat-treated samples on the other hand, re-isolation failed. The quantity of toxin remained at a level corresponding to the first period of formation. The slight fluctuations found were due to methodical error. Contamination with other moulds as well suggested that exclusively the *Fusaria* were sensitive to the applied heat exposure (80 °C).

The F-2 toxin content of the differently treated samples tended to increase over the control with progressing time. We offer two hypothetical explanations to this phenomenon.

In view of the known mutagenic effect of ionizing irradiation, it may be postulated that irradiation induced mutation towards a greater intensity of toxin production.

Table VI

Patulin production in cultures infected with *Penicillium urticae*

	I	II
Before irradiation (control)	+	++
	1/2+	1/2+
After irradiation		
Dose: 0.5 Mrad		
Dose performance: 250 krad/h	++++	+++
400 krad/h	+++	+++
500 krad/h	–	++
Dose: 1.0 Mrad		
Dose performance: 250 krad/h	++++	+++
400 krad/h	++1/2+	++
500 krad/h	–	–
Dose: 1.5 Mrad		
Dose performance: 250 krad/h	+++++	+++++
400 krad/h	++++	++++
500 krad/h	–	–

Ochratoxin A production in cultures infected by *Aspergillus ochraceus*

	I	II
Before irradiation (control)	++	+++
	+++	++
After irradiation		
Dose: 1.0 Mrad		
Dose performance: 500 krad'h	+++++	++++1/2+

Ionizing irradiation also accounts for splitting of large-molecular organic compounds. The enzymes responsible for toxin synthesis may more easily link with simpler molecules. Since the *Fusarium* strain could be re-isolated from the irradiated samples, it has been concluded that the applied irradiation parameters (1) have been unsuitable for the destruction of fusaria growing in fodder, and (2) failed to destroy the F-2 toxin present in maize kernels.

On combined application of heat and radiation treatment, the toxin content of the samples rose a short time to a relatively high level, where it persisted. We believe that heat exposure destroyed the reproductive elements of the fungus, without inactivating its enzyme system. It is known that at 80 °C enzyme activity rises abruptly and subsequently disappears. The cause

of the rising of the toxin-quantity may have been due to the following: the splitting effect of the irradiation resulted simpler compounds and these were used easier by the toxin-synthetizing enzymes of increased activity.

Experiments with *Aspergillus* and *Penicillium* spores have permitted the following conclusions:

a) Irradiation killed the major part of the fungus spores, but, owing to the enormous reproduction capacity of fungi in general, the surviving propagules may well serve as a source of re-infection.

b) We cannot exclude that the death of the *Aspergillus* spores had been due to technical error, for spores survived in a single culture.

c) Remarkably, the *P. urticae* and *A. ochraeus* cultures surviving irradiation produced 3—5 and about 2 times more toxin, respectively, than the non-irradiated cultures.

The positive response of *Penicillum* fungi to irradiation might probably be utilized in the drug industry for increasing the output of antibiotic-producing strains.

Summary

Maize kernels were infected experimentally with spore suspensions of *F. culmorum*, *A. ochraceus* and *P. urticae* strains producing F-2, ochratoxin and patulin, respectively. Samples of the Fusarium-infected maize were exposed to heat, irradiation, or heat+irradiation, while the *Aspergillus*- and *Penicillium*-infected samples were only irradiated. After an appropriate incubation, re-isolation experiments were performed, and toxin determinations and assays were carried out.

Heat treatment killed the *Fusarium* fungi, but not the *Aspergillus* and *Penicillium* cells. Irradiation accounted for depression of fungal growth, but enhanced the mycotoxin production of the radiation-resistant cultures. Combined heat and irradiation treatment was followed by an abrupt rise of toxin content, and persistence of the elevated toxin level in the exposed samples.

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CUTANEOUS SPHAEROSPOROSIS OF THE COMMON CARP FRY

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Branchial and renal sphaerosporosis of the common carp fry in Hungary, caused by *Sphaerospora carassii* Kudo, 1919 *(Protozoa: Myxosporidia)* and *S. angulate* Fujita, 1912, respectively, was reported earlier in this Journal and elsewhere (MOLNÁR, 1978, 1979).

In more recent studies, we have detected many sphaerospora stages in cutaneous scrapings obtained from young carps and investigated the condition in detail. The results are reported in this paper.

Materials and methods

Eighty 1 to 3 months old carps, and 156 3—6 months old carp fry, obtained from different pond farms, were examined above all for the frequency of renal sphaerosporosis. About 90% of the carps were mirror carps, the rest were scaled specimens. The incidence of concurrent infections was studied in impression smears of organs other than the kidney, and in scrapings from the gill and skin. The organs infected by sphaerospora stages or, if the host was small, the fish *in toto*, were fixed in 10% formalin or Bouin solution, embedded in paraffin, and cut to 3—6 μm thick sections. From *in toto* processed fish not longer than 1.5—2 cm, longitudinal sections were cut to include details of gills, skin and kidney in a single section. The sections were stained with haematoxylin and eosin, or with the Farkas—Mallory technique.

Results

Of the 80 1—3-summer carps, 16 harboured *S. carassii* spores in the gills, and 2 had *S. angulata* spores in the kidney. Histological evidence of cutaneous sphaerosporosis was obtained only later, and only this prompted us to scrutinize the cutaneous scrapings. Nine hosts, all with gill sphaerosporosis, were found to harbour the parasite in cutaneous locations as well. Renal spaerosporosis was not associated with gill or skin involvement in either case. Of the

156 3—6 months old carps 36 had a renal *S. angulata* infection, but neither gill nor skin sphaerosporosis occurred in this age group.

The spores detected in the skin were similar in shape and size to those established in the gills, but differed from those found in the kidney.

The histological examinations showed that the massive infection spread from the gill filament and gill-arch epithelium to the lining epithelium of the gill cavity, which was packed with spores and developmental stages everywhere except in the thymic area. The infection was similarly massive in the skin surrounding the branchial slit, but much milder in the areas adjoining the dorsal and abdominal fins. Spores were occasionally found in the skin of the head, exclusively in those areas where the epithelium was thick. No parasites were detected in the epithelium of the operculum and in the skin covering the posterior part of the body.

In the cutaneous areas adjoining the branchial slit, the cutaneous epithelium was uniformly infected from the stratum germinativum to the surface. Developmental stages occurred only near the stratum germinativum, but spores were detected over the entire thickness of the epithelium (Fig. 1). In the cutaneous epithelium of the head and central part of the body, spores occurred only in minor islet-like aggregations (Fig. 2). In the massively infected cutaneous regions, the structure of the epithelium became rarefied, and part of the epithelial cells showed a serous degeneration. The surface of the epithelium was covered by damaged cells and structureless cell debris.

Discussion

The present studies have shown that *S. carassii*, formerly regarded as exclusively a gill parasite, is also responsible for a cutaneous sphaerosporosis, which involves not only the stratified epithelium of the gill filaments and gill arches, but also the lining epithelium of the gill cavity and certain cutaneous areas of the body of carps. There is reason to postulate that sphaerosporosis of the gills is always associated with a cutaneous involvement, although evidence of this could be obtained only in 1 to 3 months old carps in the present study.

The histological examinations have shown that gill and skin involvement are always continuous. Since among the scaled carps included in the study noneharboured sphaerospores, it might be questioned whether the parasite could establish itself in a scaly skin at all. It also remains to be clarified how long the cutaneous sphaerosporosis might persist, for among the hosts included in the study only the less than 3 months old fry was infected with *S. carassii*. It is known from earlier studies (MOLNÁR, 1979) that the sphaerosporosis of the gill may persist longer than 4 months in infected hosts. There is reason to postulate that the cutaneous infection takes a similarly long course.

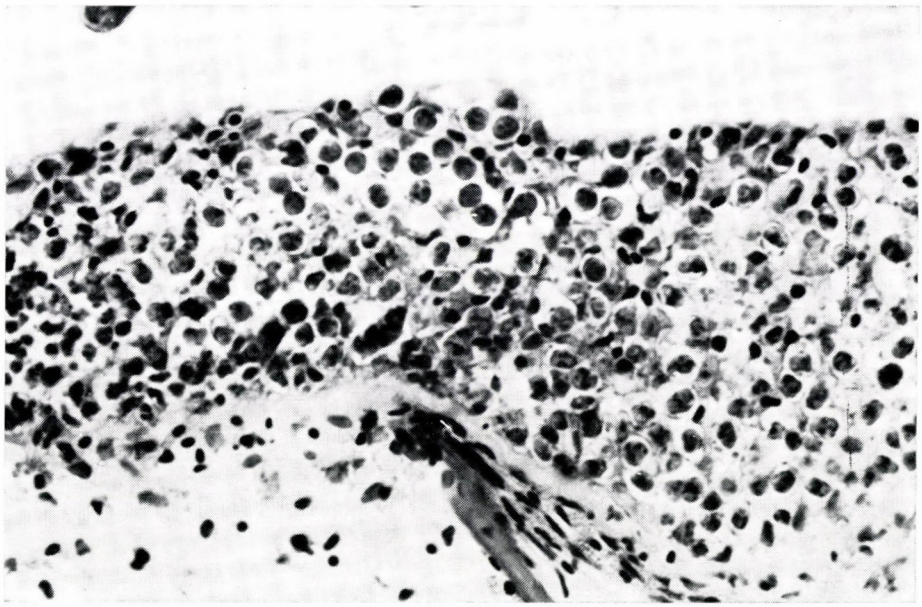

Fig. 1. Spores and developmental stages in the skin of a carp fry

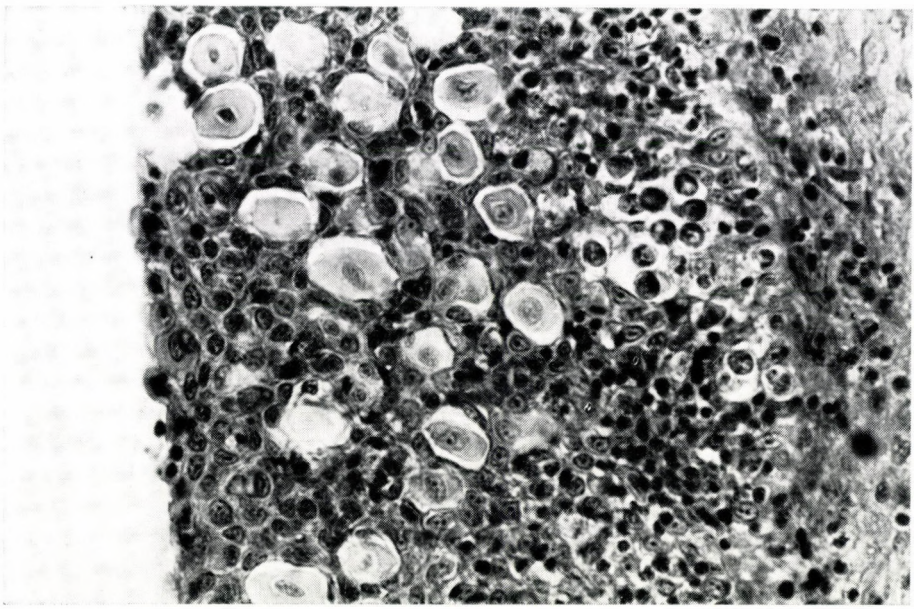

Fig. 2. Islet-like aggregations of *S. carassii* spores in the cutaneous epithelium

The morphological characteristics of the spores, as well as the epizootological and histological observations unequivocally support the conclusion that there is no causal relationship between the cutaneous sphaerosporosis and the renal *S. angulata* infection of fish hosts.

Summary

The cutaneous sphaerosporosis of the common carp fry is caused by *Sphaerospora carassii* Kudo, 1919, which also accounts for gill sphaerosporosis in the same host. Spores and developmental stages of *S. carassii* were detected in large numbers in cutaneous areas near the gills of 1—3 months old carp fry.

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EXPERIMENTAL INFECTION ON SHEEP AND PIGS WITH *ECHINOCOCCUS GRANULOSUS* (BATSCH, 1786), AND THE ORIGIN OF POUCHING IN HYDATID CYSTS

By

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It may happen to a simple hydatidosis particularly in sheep, less frequently in pig and in bovines, that a certain degree of pouching is seen in cysts recovered from organs of these animals. The cysts are not spherical and their wall is not quite smooth. Due to the high degree of pouching of the cyst wall the cyst may resemble multilocular with loculi of varying size separated by incomplete septa and communicating with a common cavity. The complicated structure of the cavity might overshadow the individual loculi inside the cyst. Frequently, these cysts are confused with a multicystic form of hydatidosis. The factors responsible for this abnormal development of hydatid cysts need to be clarified.

Certain "multilobular" forms of hydatid cysts were observed by Kuznetsov et al. (1976) in experimental direct and cross infections of sheep and pigs with eggs of *E. granulosus* (Batsch, 1786). The animals were derived from various areas of the U.S.S.R.

Materials and methods

Experiments with the pig and the sheep from the Moscow district were made at VIGIS (Vsesoyusniy Gelmint. Inst. K. I. Skryabina). Two different strains of *E. granulosus* were used in these experiments, one obtained from Uzbekistan sheep (Samarkand), the other from Ukrainian pigs (Lvov). After a successful infection of dogs performed in Moscow, eggs from the infected animals were used in these experiments.

Pieces of infected organs were fixed in 10% formol and made either into complete or incomplete histological series, using standard and specific staining methods. In order to demonstrate the shape of hydatid cavities, plastic models were made of several hydatids from a series of sections (Šlais, 1978).

Results

1. The echinococcus strain of Ukrainian pig origin was used for an experimental infection of pigs and sheep 2 and 6 months of age.

A massive infection was established in the livers and lungs of many 2-month-old pigs. At 6 months post infection (p.i.) already, typical sterile hydatid cysts, more than 25 mm in diameter, were recovered from a non-active capsule. The relatively thick cuticle (100 μm) of the cysts was in direct contact with a thick layer (150 μm) of hyaline connective tissue. A zone of chronic inflammation (30 μm) was present on the side facing the atrophic liver tissue. At 15 months p.i., similar, also sterile, cysts were surrounded by a more richly infiltrated capsule of connective tissue, and there was a continuous line of multinucleate cells on the surface of the cyst. If cysts were surrounded by an active inflammatory capsule in some of the experimental animals, the cyst never exceeded 8 mm, even at 12 months p.i., and numerous cysts were in a state of regression. In this group of hydatide, we observed neither a necrosis of their connective tissue capsule nor a demarkating, secondary, epitheloid border.

The infection experiment was less successful with sheep aged 2 months. There were a few, minute, hyaline nodules in the livers of several animals, but the nodules were difficult to identify. At 14 months p.i., a single, minute, shrivelled hydatid cyst and several nodules were recovered from the liver of one sheep.

Better results were obtained with 6-month-old pigs, in which hydatids started to develop in the liver and the lung. At 7 months p.i., the hydatids, mostly sterile, reached 15 mm in diameter. Quite often, there persisted an active tissue reaction with an epitheloid border, an exudation, and a marked lymphoid infiltration of the connective tissue capsule. However, at the same time, there were traces of dead hydatids in the form of minute nodules. At 10 months p.i., we still found large, but regressively changed, sterile hydatids. Capsular necrosis was not found in this group.

Of the 6-month-old sheep, only two became infected, and these developed pathological changes after 7 months. Ovoid granulomas in the liver and the lungs, and occasionally in the kidney and the spleen, frequently contained asymmetrical cysts showing advanced dystrophy. A necrosis of the exudate and the connective tissue capsule at the site of contact with the membrane of the parasite indicated a high degree of sensitivity of the sheep to echinococcus infection. The developing defence reaction of the organism killed the hydatids and caused their resorption which could still be observed at month 7 p.i., but not later in longer surviving sheep.

2. The echinococcus strain from the liver of the Uzbekistan sheep was used for experimental infection of pigs and sheep aged 2 and 6 months, respectively.

Fig. 1. Multilobular cyst from below the pleura of the lungs of a sheep at 12 months p.i., diameter 10 mm, following an infection of a two-month-old sheep with the Uzbekistan echinococcus strain of sheep origin. Pouching of the cyst wall seen even below the pleura of the lungs. Macrophotograph

Fig. 2. Cyst of Fig. 1 with the top layer cut off. Note extensive loculi separated from each other by narrow septa of necrotic connective tissue. Macrophotograph

Fig. 3. Model of the cyst cavity showing the bottom half of the cyst from Fig. 2, viewed from below. The parenchyma of the lungs is entered by numerous sacculi. Green colour in Fig. 8A indicates sites of contact with an active tissue reaction. Macrophotograph

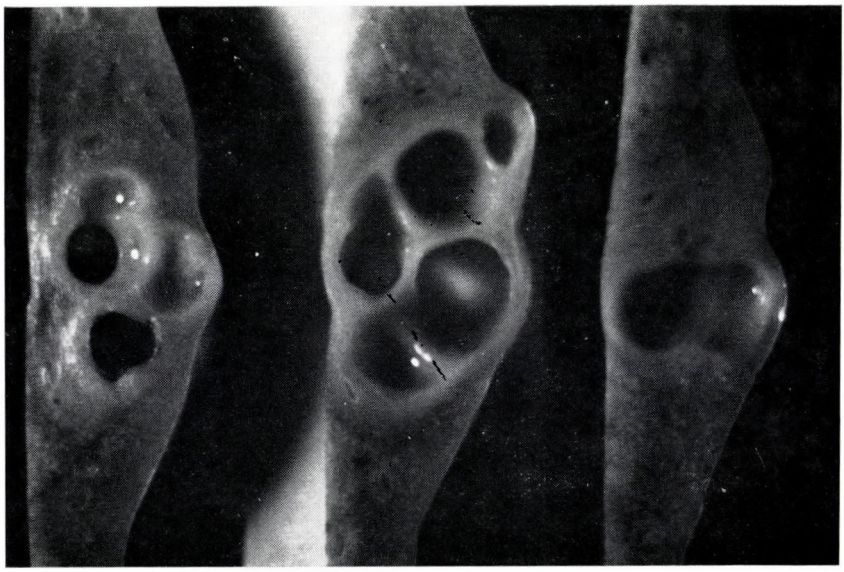


Fig. 4. Three sections through a multilobular cyst from below the pleura of the lungs apparently formed by an accumulation of several loculi. Longest diameter 12 mm. Origin of the material identical to that in Fig. 1. Macrophotograph

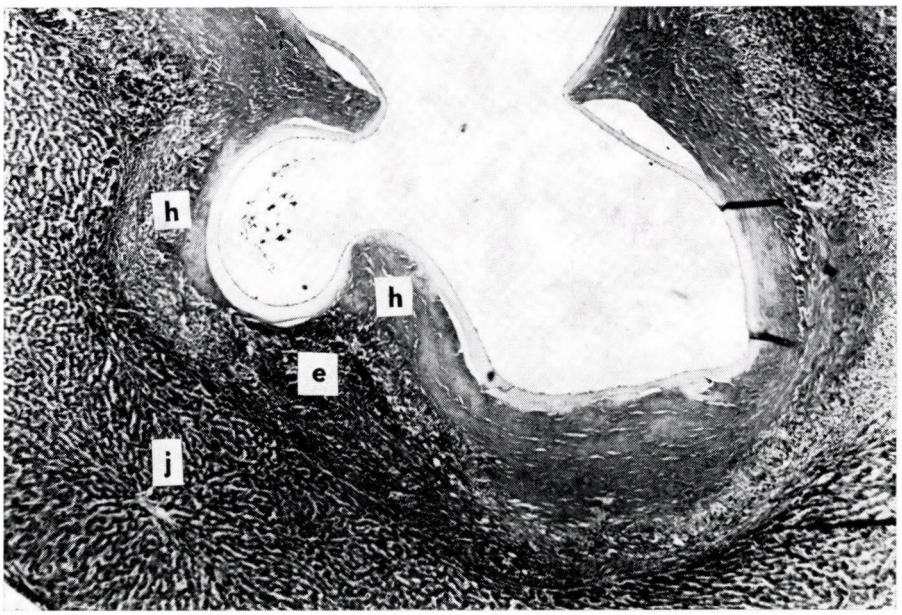


Fig. 5. Multilobular cyst in the liver. Cyst enclosed in a zone of fibrous, necrotic, connective tissue, from the sheep at 12 months p.i. of infection with the ovine echinococcus strain. Pouching of the cyst wall partly covered with a necrotic layer (h), partly still adherent to the zone of active tissue reaction (e). The site shown in Fig. 8 E. J, liver tissue. Haematoxylin-eosin (×100)

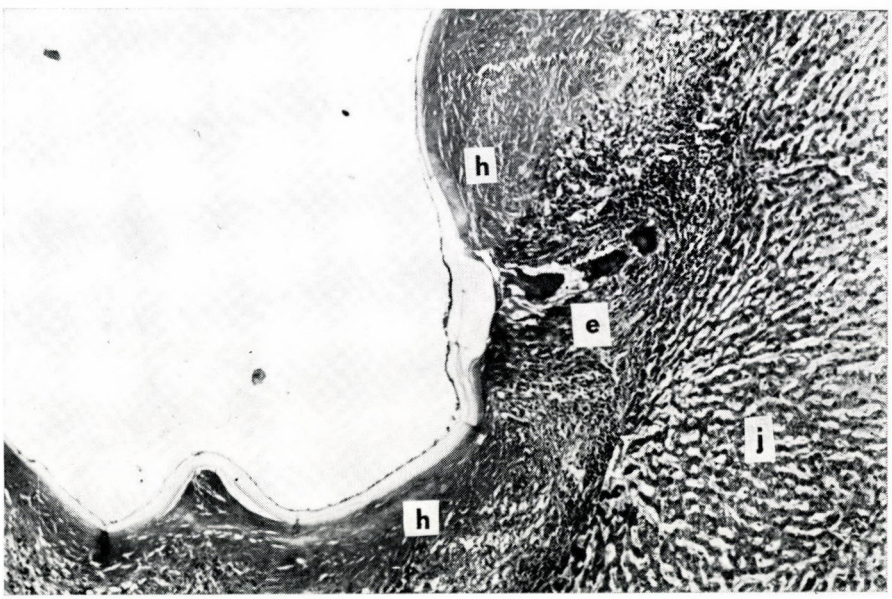


Fig. 6. Cyst wall from Fig. 5. At one site an active, epitheloid and giant-cell reaction characteristic in organization (e). The remaining parts of the wall adjoin a mainly necrotic, newly-formed connective tissue (h). Site figured in Fig. 8 F. Haematoxylin-eosin (×100)

A partial experimental hydatidosis could be established in the liver and the lungs of 17 two-month-old pigs infected with the ovine strain of *E. granulosus*. In several animals, the infection was suppressed in the first months, judging from the presence of resorptive nodules, while in others hydatids grew steadily to reach maximum diameter of 10 mm after 7 and 8 months. In several pigs, remarkable, fertile cysts (maximum size 40×60 mm) developed in the liver at 10—12 months p.i. Adjacent to their surface was a layer of multinucleate cells, and their connective tissue capsule showed a heavier inflammatory infiltration. The proliferation of a newly-formed connective tissue in regressively-changing cysts was increased in comparison with that following an infection with the porcine echinococcus strain.

In the group of sheep aged 2 months, the number of infected animals was 21. The resorptive nodules present from the first to the sixth month were characterized by a necrosis which was spreading from their centre to the connective tissue capsule. After 8 and 12 months, there was a multiple or even massive infection in the lungs and the liver, and occasionally also in the kidneys and the spleen. At months 16 and 26 p.i., regressive features in similar granulomas were more marked except for a single pulmonary cyst, which was fertile in spite of its modified shape after the longest time of exposure (26 months). Granulomas (longer axis 11 mm) possessed an increased number of cystic cavities (Fig. 4) at month 12 p.i. Several of these with better developed and more numerous loculi and of a more compact structure were seen to arise considerably above the surface of the organ (Fig. 1). Granulomas seated deeper in the organs, mainly in the liver, were more racemose in their appearance; loculi in the lungs were more spacious (Figs 2, 3). Our models of the cyst cavity demonstrated a single cystic structure with a common cavity forming secondary pouches which again produced minute buds (Fig. 8). The hydatids were surrounded by a capsule consisting of a necrotic connective tissue with several calcifications. The host tissue was shown to react only to later-produced minute pouches representing the last zone of growth of the cyst, by bringing forth an epitheloid and particularly giant-cell response adjacent to the remarkably increased lymphoid tissue at the periphery of the connective tissue capsule.

The experimental infection of 6-month-old pigs resulted in a massive infestation with hydatids reaching 10 mm in diameter at 7 months p.i. The capsule was heavily infiltrated with eosinophils diffusing to the surface of the parasite. There was also a remarkable fibroblastic proliferation and a production of collagenous fibrils at the site adjacent to the infiltrated tissue. A striking feature was the incidence of necroses of the connective tissue capsule bordered by an eosinophile infiltration and an epitheloid rim. In the following months of the experiment, hydatids were found in a state of regression, and there was a steady progress in all capsular changes.

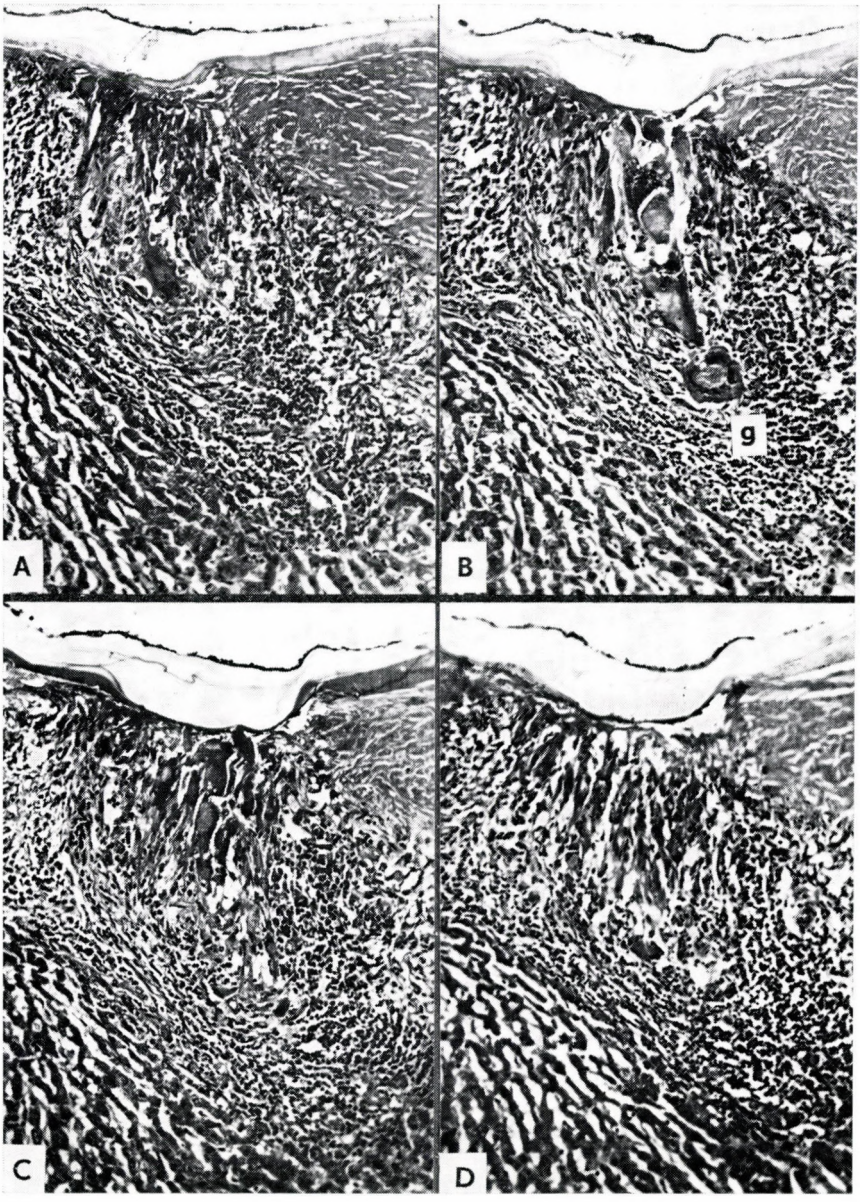


Fig. 7 A—D. Four subsequent sections through the zone of an epitheloid and giant-cell reaction figured in Fig. 6. The cuticular membrane is swollen at the site of its contact with the cellular reaction. A giant cell of the type of a Langhans' cell can be seen inside the accumulated lymphoid tissue (B-g). Haematoxylin-eosin (×160)

In the sheep infected at the age of six months, indistinct resorptive nodules appeared in the liver at 7 months p.i., but hydatids could not be found.

Evaluation of results

Pigs from the Moscow district were successfully infected at the age of 2 and 6 months, with an echinococcus strain of Ukrainian pig origin and a strain of Uzbekistan sheep origin.

According to the size of hydatids and their capsule, typical cysts developed in 2-month-old pigs infected with the porcine strain. However, the hydatids were invariably sterile and showed regressive changes similar to those observed in pigs infected when 6 months old. The tissue reaction was often active, mainly at the start of regressive changes in the parasite. Contrary to the successful infection of pigs aged 6 months with the ovine strain, the connective tissue capsule was not necrotic. The porcine strain produced an infection also in two-month-old pigs, resulting in the finding of a fertile hydatid (40×60 mm) as early as 12 months p.i. However, there was always a heavier reaction to the ovine strain, with an incidence of necroses of the connective tissue capsule, an increased infiltration and an exudation of eosinophils often connected with regressive changes in the hydatid and accompanied by an even more marked proliferation of the connective tissue.

A heavy and even massive infection in 6-month-old sheep was caused by the porcine strain; similar results were obtained with the ovine strain in two-month-old sheep. In the remaining groups, but also in many animals

Fig. 8 A—F. Models of the cavity of multilobulate hydatid cysts of *E. granulosus*. Explanation of colours: White — surface covered with necrotic exudate or connective tissue. Green — active cellular reaction. Blue — calcified foci. Yellow — commencement of shrivelling in older parts of the cyst

A, Multilobular cyst from the lungs of a sheep infected at the age of two months with an ovine echinococcus strain from Uzbekistan. Situation at 12 months p.i. Cavity model viewed from below, showing the bottom half of the cavity (object 10 mm in diameter) with a certain degree of pouching in the cyst wall growing into the lung parenchyma

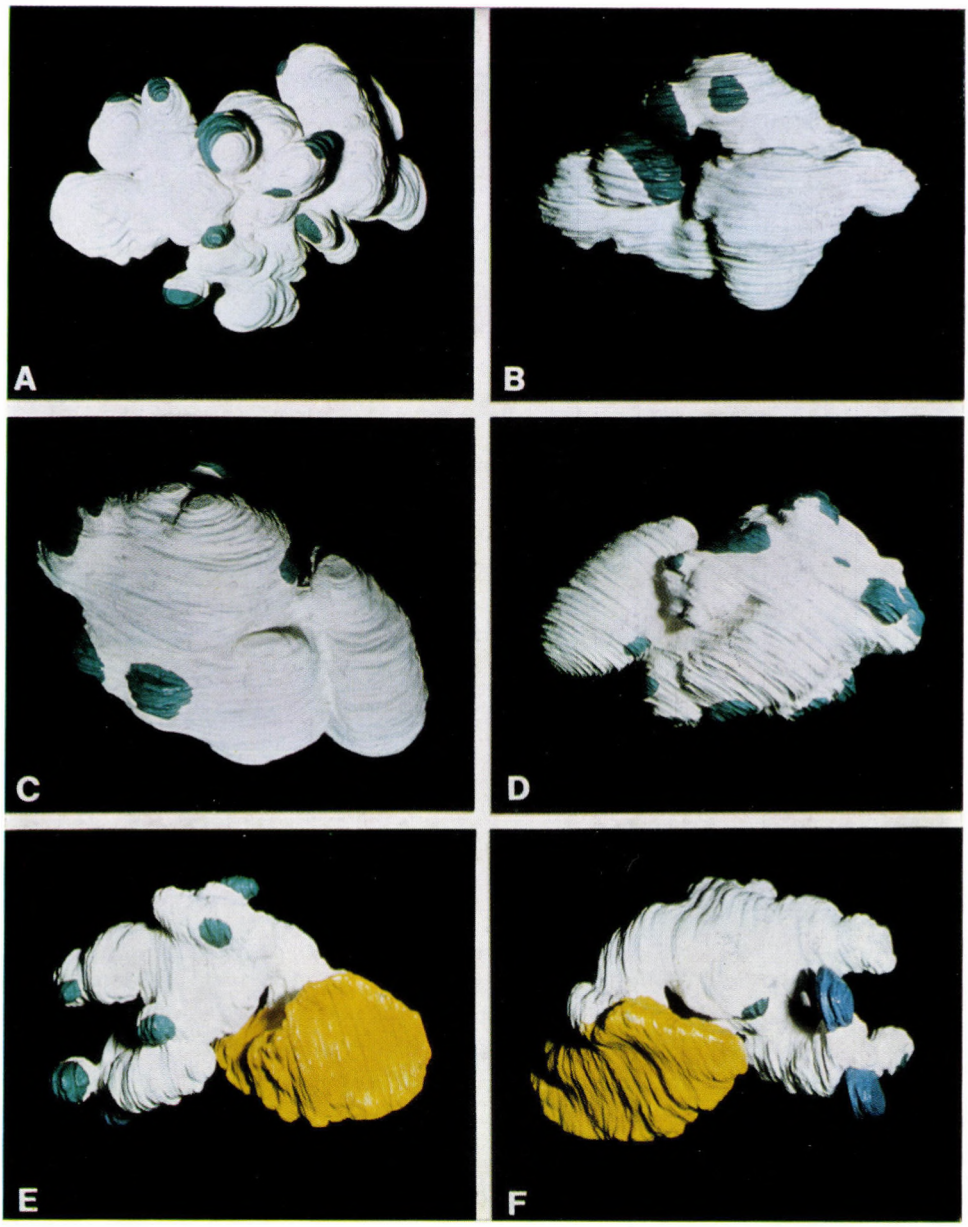
B, Multilobulate cyst located in deeper parts of the lung parenchyma at month 14 p. i. following an infection of the sheep with the ovine strain. The longer diameter of the object is 8—10 mm. The model of the cavity shows a division of the original cavity into two branches (left), in which further sacculi originate

C, Lobular cyst from the lungs of sheep infected with the porcine echinococcus strain. Situation at 14 months p.i. The longer diameter of the object is 6—8 mm. Old sacculi in the cyst wall enclosed by a necrosis

D, Model in Fig. C viewed from the opposite side. Newly-originating pouching in a more recent and more expanded part of the cyst

E, Cyst from the liver of the sheep infected at the age of 2 months with the ovine strain. Month 12 p.i. The primary subcapsular cyst (yellow) is shrivelling, measuring 3 mm in diameter. At the left, below centre a proliferating expansion (green) seen in Fig. 5

F, Opposite side of the cyst in Fig. E. The original, shrivelling cyst shades in a new, further proliferating part. Minute contact with the tissue (green) hit in Figs 6 and 7. To the right: Two calcified foci checking the terminal zone of growth of the cyst


A
B
C
D
E
F

of the groups under consideration, the presence of resorptive nodules indicating that the parasite had died in the early phase of its development, was frequently observed. The high sensitivity of the sheep to echinococcus antigens was reflected in the development of a necrosis around these cysts. This was responsible for specific changes even in parasites which had started to develop. Of particular frequency were these changes in 6-month-old sheep infected with the porcine strain of the echinococcus.

A capsule of newly-formed connective tissue developed around the parasite. This was adjacent to its cuticular membrane. The wide zone of connective tissue was necrotized; unaffected parts of the connective tissue were frequently lined by a secondary, demarkating, epitheloid rim. In several parts, where the capsule of connective tissue was not adjacent to the surface of the parasite, the cuticular membrane was either in direct contact with the epitheloid rim or, more frequently, with accumulated multinucleate giant cells produced focally in these parts of the connective tissue capsule (Figs 5, 6). There the cuticular membrane of the hydatid was ticker than in parts directly adjacent to the necrotic connective tissue. At the periphery of the giant cell reaction, the newly-formed connective tissue contained flat, remarkable, lymphatic follicles produced by a secondary accumulation of lymphoid tissue which in histological sections obscured peripherally foci of giant cells. We often observed germinal centres. This abundant lymphoid tissue was present also between the individual sacculi giving the granuloma its ovoid form. Follicles were also clearly visible inside the connective tissue capsule.

The parasite was in a state of advanced dystrophy. In some granulomas, we found only resorbing necrotic foci frequently separated from each other by a septum of newly-formed connective tissue. Our model of the cyst cavity showed communications between individual loculi.

In the group of 2-month-old sheep infected with the ovine strain, a similar host reaction did not impede the development of deformed hydatids, of which some even were fertile. Apparently, this strain was better-adapted to defence reactions of its host, the sheep. The cyst, avoiding obstacles to its growth such as sites of an accumulation of connective tissue which might have easily become necrotic, proceeded in its growth along the line of the least resistance. Frequently, pouches in the cyst wall surpassed in size that of the original cyst. At first, they were in contact with a locally confined epitheloid and giant-cell border and functionally associated with a follicular, lymphoid accumulation containing germinal centres. The parasite responded to the tissue reaction with swelling of its cuticular membrane, later it was disrupted by giant cells. This reaction checked the development of pouches in the cyst wall until they were covered completely by a necrosis of the cellular rim and a connective tissue proliferating from the outside. Of importance was the finding of a local accumulation of cells and their mitosis in the germinal layer

of the wall, because it demonstrated an increased proliferation of the cyst wall at the site of its pouching. Generally, the strip of multinucleate cells continued from the pouching wall to the lymphoid follicle. The cells were elongate cytoplasmic structures and their nuclei were concentrated at that part more remote from the parasite except for one larger cell situated close to the germinal centre of the follicle which in the peripheral position of its nuclei resembled Langhans' cells (Fig. 7). It was possible to distinguish the checked growth of proliferated pouching in the cyst wall even in parts of an older necrosis. After the parasite had died, a resorptive cellular reaction liquidated necrotic and often calcified foci, and the granuloma started to cicatrize.

Discussion

Pouching in the wall of the hydatid cyst of *E. granulosus*, particularly in the pig, is caused by an uneven resistance of atrophying liver- and lung tissues to the growing cyst (CAMERON, 1927). The result is an asymmetric shape of the cyst ranging from a simple pouching to the origin of multilocular cysts. Pouching in cysts from an experimental hydatidosis in the pig was observed by DEW (1925) in the first month p.i. He ascribed their remarkable incidence in the liver in the 5th month p.i. rather to the purely mechanical resistance of thick-walled vessels and gall-ducts interfering with the growth of the cyst than to a true budding of the cyst wall. He suggested even a coalescence of similar multilobular cysts. HUTCHISON (1960) demonstrated cysts with characteristic outlines below the surface of the liver capsule in a spontaneous massive liver hydatidosis of the dog. The structure of very old cysts (ŠLAIS and VANĚK, 1960) and very young cysts (ŠLAIS in press), was studied on an extensive material obtained from spontaneous hydatidosis. Basing on the course of a dissimilar pressure atrophy of individual liver lobuli, it was possible to demonstrate the growth of the individual pouches and the persistence of an active trizonal tissue reaction in their capsule. Pouching of hydatids in equines was demonstrated by BENEX (1968) in histological sections; he explanated fragments of the germinal membrane of multilocular cysts and observed an active pouching of a cyst growing *in vitro*.

CAMERON and WEBSTER (1966) drew attention to apronounced zone of fibrous connective tissue observed in an experimental hydatidosis in the sheep. The zone was dense, thick, avascular and necrotic. It developed earlier than in the pig. DEW (1953, 1958) maintained that resistant structures in the host were responsible for an asymmetrical growth of hydatid cysts in both the sheep and the pig, and added that the incidence of multilocular cysts of different sizes occurred more frequently in the sheep, particularly in older animals, than it did in the pig. He drew attention to the fact that the fibrous capsule

started very early to become dense and thick, and suggested that this might be an early process preventing a symmetrical enlargment of the parasite.

The difference between a multilocular and a multicystic form of hydatidosis is indistinct in both the sheep and the bovines although in the latter the shape of the multicystic form is most characteristic. DEW (1958) himself admitted that this form is frequently called "echinococcus multilocularis". In order to avoid confusion, VANĚK (1980) suggested to use the term "lobular" for cysts with a limited number of pouches, while "multilobular" for those with numerous communicating sacculi. It is most difficult to distinguish communications between individual loculi of a multilobular cyst in sections partly because loculi may be separated while sections are cut (Fig. 4), partly, the parasite and the whole granuloma might have been changed by an advanced dystrophy and regression. However, cavities of different sizes, completely separated from each other by connective tissue septa are regarded as typical of the multicystic form of hydatidosis. Therefore, several authors regarded it as a degenerated larva of *Alveococcus multilocularis* at an advanced state of regressive changes in its tissue. It is obvious that a differentiation of the two forms needs further comparative studies of the material. Of importance is the fact that our material under study consisted of multilobular forms at different stages of their development recovered from sheep either infected with an echinococcus strain of sheep- or of pig origin. However, from the pig infected with the same echinococcus strain of sheep origin, we obtained spherical cysts and the tissue reaction although active at various stages proceeded without a remarkable proliferation of the fibrous connective tissue. Therefore, the origin of multilobular cysts in the sheep occurred under the influence of the tissue reaction of the host.

Summary

Sheep and pigs from the Moscow district (U.S.S.R.) were infected with eggs of *Echinococcus granulosus* (Batsch, 1786) obtained from donors after an artificial infection with an echinococcus strain of Ukrainian pig origin and a strain of Uzbekistan sheep origin. Hydatids obtained from the pig infected at the age of 6 months with either of the strains were invariably sterile. Fertility was attained by hydatids from an infection of 2-month-old pigs with the ovine strain. A heavy and even massive infection developed in 6-month-old sheep infected with the porcine strain and in 2-month-old sheep infected with the ovine strain. In the latter case, even a fertile cyst was recovered in month 26 p.i. Multilobulate cysts developed in all successfully infected sheep but mainly in those infected with the ovine strain, while in the pig the cystic structures were symmetrical in shape and there was a corresponding tissue reaction. Due to the considerable sensitivity of the sheep to antigens of the echinococcus, the fibrous connective tissue became heavily thick, avascular and necrotic, thus, influenced the shape of the growing cyst. Its wall formed saccular expansions which at first were adherent to the active epitheloid cellular reaction, later were included in the necrotizing zone. Thus, the tissue reaction of the host was responsible for the origin of multilobular cysts in the sheep.

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A LIVE ATTENUATED VIRUS VACCINE AGAINST SHEEP POX

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Sheep pox is one of the longest-known infectious animal diseases. Formerly, it was responsible for devastating outbreaks in sheep flocks. Attempts at its control had long preceded the identification of its causal agent. Initially, protective treatment, called "ovination", was carried out by transfer of pustular contents from diseased to healthy sheep, but this prophylactic measure was too hazardous, for it occasionally accounted for as much loss as the outbreak itself. An improved form of ovination, *i.e.* the use of diluted pustular material, usually caused only mild untoward reactions. This was followed by the use of inactivated preparations, which contained virus treated physically and chemically to depress its infectivity without affecting its antigenicity. Attenuation was attempted by propagation of the virus in different animal species (goat, rabbit, chicken embryo), but the immunogenicity of the adapted strains was inconsistent.

In Europe, an aluminium hydroxide gel-adsorbed, formalin-inactivated sheep pox vaccine containing an antigen prepared in sheep according to Borrel (1903) has been most widely used; the Hungarian variant of this vaccine was issued under the trade name "Phylaxovina" (Manninger, 1948). Controlled vaccination programmes and other veterinary measures have resulted in the eradication of this dangerous sheep disease from Europe, but it still occurs in Asia and Africa.

Introduction of the tissue culture techniques in virological work opened new ways in virus vaccine production. The propagation of sheep pox virus in ovine testicular tissue was originated by Bridré (1935). Aygün (1955) demonstrated that the virus lost its virulence for sheep after 12 subsequent transfers in embryonic ovine testicle or lung cell cultures. Plowright and Ferris (1958), as well as Cilli and Baldelli (1957) and Cilli (1961) conducted detailed investigations into the properties of tissue-culture-propagated sheep pox virus, and on the basis of their findings. Ramyar (1965) and Ramyar and Hessami (1969) were able to attenuate a virus strain by at least 20 serial transfers so that it lost its infectivity for sheep completely, but retained its full immunogenicity. From the attenuated strain, the last-cited authors prepared a potent live vaccine by a very simple technique. Preparation of a similarly potent live vaccine from a tissue-culture-adapted strain was reported by Mateva-Pankova et al. (1974).

Development of a potent live vaccine for protection of the flocks in Mongolia against the sheep pox virus strains indigenous in the country is reported in this paper.

Materials and methods

Experimental animals

Landrace and Merino sheep produced in Mongolia were used in the experiments. The animals were derived from a "clean" herd, kept in a region where no outbreak of sheep pox had occurred for 10 years, and the "cleanness' of each experimental animal was ascertained by preliminary serological tests.

Virus strains

The virus strain designated "Perogo", originally isolated in Algeria and adapted to ovine testicle cell culture in Bulgaria, was used. We propagated the strain in two types of cell culture, derived from ovine testicle and ovine kidney, respectively; it replicated readily in both. For challenge the strain MH, which has been used as vaccine strain for about 30 years, and the strain DAB, isolated from an outbreak two years ago, were employed.

Complement fixation (CF) test

Both macro- and microtechniques were used. The sheep erythrocyte concentration was standardized by spectrophotometry for an extinction of $HD_{100} = 0.2$. Reactions obtained with the macrotechnique were also read by spectrophotometry.

Cell culture

Testicle cells derived from 3—4 months old lambs were used for tests and vaccine production. The cultures were prepared in Roux flasks, using Hanks' solution enriched with 10% calf serum as growth medium. The cultures were inoculated with the virus after outgrowth of the confluent monolayer, which took 2—4 days. The medium was decanted before inoculation.

Propagation of the virus

Reconstituted freeze-dried virus was used as inoculum at the dose level of $3—4 \times 10^4$ $TCID_{50}$ per flask. The virus was allowed to adsorb for 1 h before the VM3 maintenance medium was added. Light-microscopically visible CPE usually appeared after 3 days, and it involved 80—90% of the culture between days 5 and 8. The virus was harvested at this stage. The flasks were cooled to —20 °C, and the frozen cells were subsequently thawed in a water bath at 37 °C. Alternate freezing and thawing was repeated three times to promote virus release from cells. The released virus was then titrated.

Results

Table I shows that a substantial part of the virus persisted in intracellular localization despite repeated freezing and thawing. For this reason, the virus-containing cell suspension intended for vaccine production was not centrifuged, to prevent virus loss; there was no hazard of an allergic or any

Table I

Extra- and intracellular virus content of tissue culture infected with attenuated sheep pox virus

Sample No.	1	2	3
	log $TCID_{50}$		
1	4.5	5.83	5.5
2	3.8	4.5	4.8
3	4.7	5.8	6.0
4	4.3	5.7	5.83

Infected cell culture was frozen and thawed three times, than it was centrifuged at 2000 g for 20 min. 1, supernatant; 2, sediment suspended in one-fifth of the original volume; 3. non-centrifuged suspension.

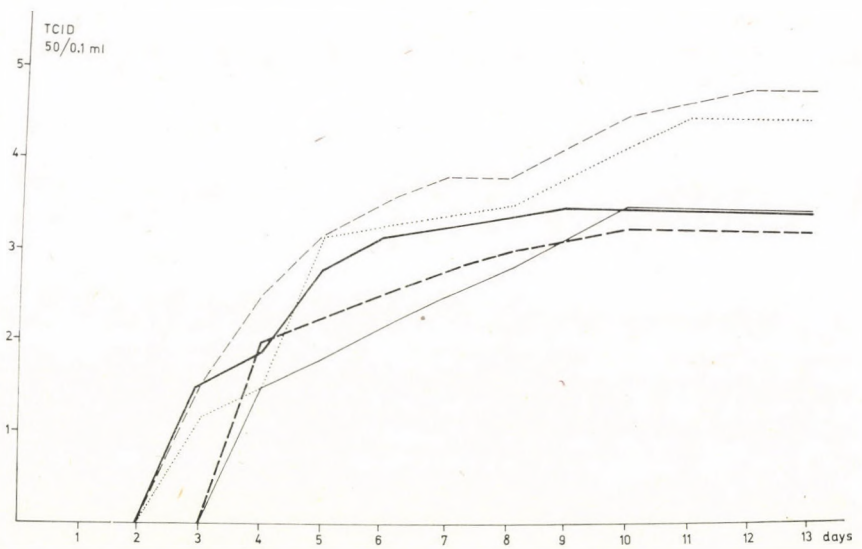

Fig. 1. Kinetics of multiplication of attenuated sheep-pox virus strain in primary sheep-testicle tissue culture. ——— supernatant; – – – – – sediment; ····· non-centrifuged suspension; ——— non-centrifuged suspension (stored 18 hrs at 24 °C); — — — freeze-dried vaccine

other untoward effect, for the cells and cell debris retained in the vaccine material originated from homologous tissue. Possible changes in the virus titre were examined in the function of time. Figure 1 shows the kinetics of virus suspensions titers. The virus was subjected to some common resistance tests, results of which are presented in Table II.

Table II

Heat- and pH-resistance of the attenuated sheep pox virus

Sample No.	Treatment			log $TCID_{50}$/ml
1	pH 7.4	50 °C	30 min	4.5
2	1*M* $MgCl_2$	50 °C	30 min	0
3	pH 3	37 °C	60 min	0
4	pH 7.4	37 °C	60 min	4.5
5	control			5.5

Table III

Influence of additives on the freeze-drying of attenuated sheep pox virus

Sample No.	Protective colloid	log $TCID_{50}$/ml
1	1% peptone + 2.5% glucose	3.13
2	20% cow's milk	3.46
3	2.5% lactalbumin hydrolysate	2.80
4	Bacto-casein	3.46
5	Control (before freeze-drying)	3.13

Among the protective colloids tested in freeze-drying (Table III), milk and bacto-casein proved to be the most practicable. As milk was readily available, all three vaccine batches were freeze-dried with it. Peptone + 2.5% glucose, also tested as protective colloid, made possible the preservation of the virus titre, but yielded a crumbling lyophilization product, while with milk and bacto-casein a neat array of needle-like crystals was obtained. Comparison of the pre- and post-lyophilization titres of the three vaccine batches (Table IV) have shown that loss of virus titre by freeze-drying can be fully prevented by the use of an appropriate protective colloid (skimmed milk). It is a general requirement that attenuated virus vaccines should contain at least 1000 $TCID_{50}$ virus per dose. This requirement should be taken into consideration at vaccine dilution; 1000 $TCID_{50}$ virus should be present in each ml of

Table IV

Freeze-drying of attenuated sheep pox virus in a medium containing 20 % skimmed milk

Sample No.	Passage No.	log. $TCID_{50}$/ml	
		before freeze-drying	after freeze-drying
1	3	4.16	4.05
2	3	4.5	4.5
3	4	5.5	5.5

Table V

Antigen contents of attenuated sheep pox virus stored for 9 days at different temperatures

Storage temperature	Complement fixing units	Antigen loss per cent
−20 °C	95	0
− 6 °C	83	13
+ 4 °C	85	11
+24 °C	44	54
+37 °C	25	74

The virus stored at −20 °C is taken as having not lost antigenic value during storage.

the diluted material, for the standard dose of the sheep pox vaccine is one ml by the s.c. route.

The sheep pox virus strain was also tested for the thermostability of its CF antigen. Aliquots of the virus suspension were stored at five different temperatures for 9 days and then titrated by the CF test (Table V).

Testing for safety

The safety test was carried out in four sheep (two of each breed) which were treated with different doses of the vaccine, intradermally (i.d.) or subcutaneously (s.c.) (Table VI). A non-vaccinated animal was used as control.

The temperatures of the vaccinees were taken twice daily on the day of vaccination and during the subsequent observation period. Each value in Fig. 2 represents the mean value of the morning and afternoon readings on the indicated day. The slightly elevated temperatures found in the initial period may be attributed to overheating of the sheep house.

Table VI

Safety test of attenuated sheep-pox vaccine in two sheep breads

Sheep No.	Breed	Vaccine		No. of injection sites	No. of infectious units administered	
		dose	application		per site	total
5390	Merino	2 ml (undiluted)	id	10	6 324	63 240
5391	Mongolian landrace	2 ml (1 : 10 dil)	id	10	632	6 324
5311	Merino	5 ml (1 : 10 dil)	sc	1	15 810	15 810
5364	Mongolian landrace	5 ml (1 : 10 dil)	sc	1	15 810	15 810

Four days after vaccine treatment, sheep No. 5390 developed hyperthermia (40—41 °C), and pocks 10—15 mm in diameter appeared at the injection site. On the next day, the diameter of the local eruptions increased to 15—20 mm, and the body temperature persisted above 40 °C. Then, the pocks

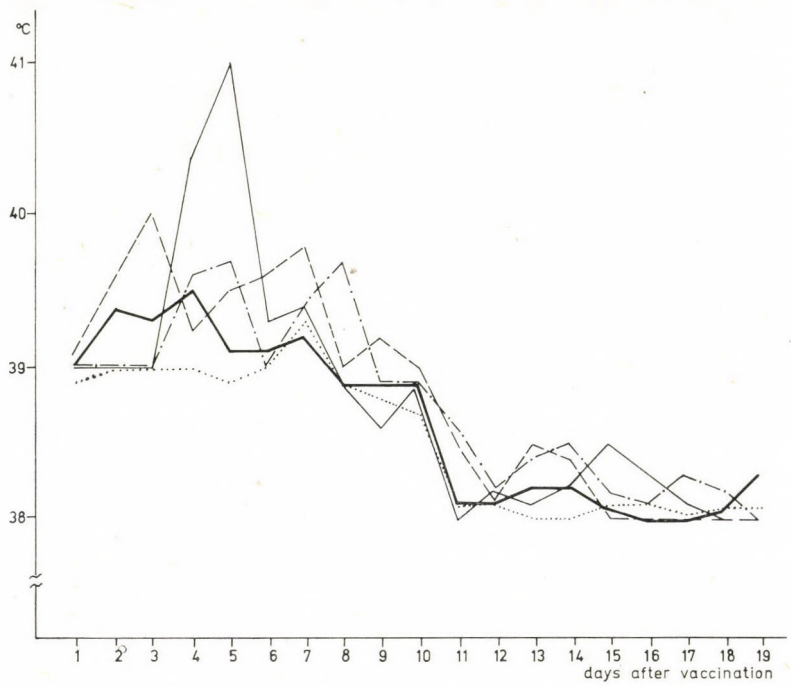

Fig. 2. Body temperature of sheep vaccinated with attenuated sheep-pox vaccine in the course of safety test. ——— sheep No. 5390; – – – – – sheep No. 5391; ····· sheep No. 5364; ——— control

ceased to grow further and the temperature returned to the level shown by the control animal.

Sheep No. 5391 showed temperature rise to 39—40 °C, and developed eruptions 5—7 mm in diameter 4 days after the treatment. The eruptions continued to grow for another day, to 8—10 mm; the body temperature ranged between 39.0 and 39.2 °C for another three days, then returned to normal, together with the temperature of the control sheep.

Sheep No. 5364 had a mild febrile course, with a maximum temperature of 39.8 °C for three days beginning on the fourth day after vaccination. Simultaneously with these animals, further 8 sheep were treated s.c. with the normal vaccine dose (1 ml); these vaccinees, too, developed a mild temperature rise, not reaching 40 °C in any case, from the fourth post-vaccination day on. Four non-vaccinated contacts, kept together with the eight vaccinees, developed neither a local nor a general reaction, and had a normal body temperature throughout.

The safety tests were evaluated 17 days after vaccination, with the following results.

a) Sheep No. 5390, treated i.d. with 80 doses of vaccine (1 ml indiluted = $= 10^{4.9}$ $TCID_{50}$/ml), developed no cutaneous lesions in areas other than the injection sites, and the eruptions developed in 10 sites were scarcely palpable at the time of evaluation.

b) Sheep No. 5391, given 8 doses of vaccine i.d., showed no indication of the eruptions which had appeared at 10 sites, nor any other change in any other cutaneous area.

c) Sheeps Nos 5311 and 5364 developed neither local nor general symptoms in response to subcutaneous administration of 20 doses of vaccine.

d) The eight sheep treated s.c. with a single dose of the vaccine developed a mild temperature elevation which lasted a few days, but no other change.

Potency testing

All vaccinees and four non-vaccinated (control) sheep were challenged with the virulent strain MH or the wild strain DAB 17 days after vaccine treatment. In most cases, one of the two challenge strains was titrated i.d. in vaccinees and controls, the titre was assessed in each case, and the titre difference between vaccinees and controls was calculated for each immunized animal as the number of infectious units against which protection was conferred by the vaccine. For titration each dilution was administered at 5 injection sites, always in 0.2 ml volume. The results of challenge with the strain MH are shown in Table VII.

Table VII

Potency test of attenuated sheep pox vaccine in vaccinated sheep challenged subsequently i.d. with the virulent MH strain

Sheep No.	Breed	Vaccine		Challenge strain	Protection against inf. units
		dose	application		
5373	Mongolian landrace	1 ml	sc.	MH	> 3 162
5714	Mongolian landrace	1 ml	sc.	MH	> 3 162
5303	Merino	1 ml	sc.	MH	>12 589
5336	Merino	1 ml	sc.	MH	4

Table VIII

Potency test of attenuated sheep pox vaccine in vaccinated sheep challenged subsequently intratracheally with the virulent DAB strain

Sheep No.	Breed	Vaccine		Challenge strain	Protection against inf. units
		dose	application		
5317	Mongolian landrace	1 ml	sc.	DAB	>50 000
5334	Merino	1 ml	sc.	intratrach.	>50 000
5389	Mongolian landrace	control	—		diseased

Table IX

Resistance test carried out in sheep vaccinated with different doses of attenuated sheep pox vaccine by means of challenge with virulent MH and DAB strains

Sheep No.	Breed	Vaccine		Challenge strain	Protection against inf. units
		n-fold dose	application		
5390	Merino	80	id.	DAB MH	>100 000 > 12 589
5391	Mongolian landrace	8	id.	DAB MH	>100 000 > 3 162
5364	Mongolian landrace	40	sc.	DAB MH	3 162 > 3 162
5311	Merino	40	sc.	DAB MH	>100 000 12 589

Two vaccinees and one non-vaccinated sheep were challenged intratracheally with the strain DAB, at the dose level of 50 000 ID_{50} per animal. The control animal developed the first pocks scattered in the head skin 13 days after infection and their entire skin area became involved within further two days (Table VIII).

The four sheep used in the safety test were challenged with both strains, having applied strain MH to one side and strain DAB to the other side of the body. The results are shown in Table IX.

Evaluation of the potency tests has permitted the following conclusions.

a) The tissue culture-derived pox vaccine protected, on the average, against 4719 infectious units of strain MH (on i.d. challenge);

b) the same vaccine protected against 50 000 infectious units of the DAB strain, in intratracheal challenge (which, in our opinion, simulates best the natural infection).

c) The animals treated with multiple doses of the vaccine for the purpose of safety testing proved to be protected against as many as 100 000 infectious units despite simultaneous infection with both challenge strains.

The examined two breeds (Mongolian Landrace and Merino) did not differ from each other in respect of immune responsiveness.

Conclusions

The great potency of our sheep pox vaccine preparation has been proved. Vaccination with 1000 infectious units of the attenuated strain conferred a firm protection on sheep against the virulent pox-virus strains MH and DAB.

The preparation is innocuous; only the animals given 80 or 8 doses i.d. developed a mild, temporary local reaction, which, however, neither involved further cutaneous areas nor spread to susceptible animals (controls) by contact. On subcutaneous administration, the way prescribed for routine vaccination, even a twenty-fold overdose failed to elicit either a cutaneous local reaction or a general febrile reaction.

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Summary

A live attenuated vaccine was prepared from a sheep-pox virus strain originally isolated in Algeria, and attenuated by serial passages in cell culture. The vaccine proved to be innocuous for both Mongolian Landrace and Merino type sheep; only multiple doses elicited a mild cutaneous reaction and temporary hyperthermia on intradermal administration, but they developed neither local nor general side effects by the subcutaneous route. The sheep treated with the recommended vaccine dose (1000 infectious units per animal) developed a firm immunity which protected them against challenge with the virulent sheep pox virus strains MH and DAB.

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DETECTION OF NEUTRALIZING ANTIBODIES TO GENUINE RABBIT ADENOVIRUS IN RABBIT ANTISERA TO HUMAN ADENOVIRUSES

By

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In 1975 we isolated, for the first time in the world, an adenovirus from rabbits. The isolate was later identified as a genuine rabbit adenovirus (Bodon et al., 1979; Bodon and Prohászka, 1980). We reported earlier that our rabbit adenovirus isolate designated as RA-1 was neutralized by certain pools of rabbit type sera to human adenoviruses. It is known that Lange et al. (1968) demonstrated fluorescent antibodies in control sera obtained from clinically healthy rabbits.

Experiments conducted in this Institute to clarify the specific or non-specific nature of the neutralization of RA-1 are reported in this paper.

Materials and methods

Sera. Hyperimmune type sera raised in rabbit or horse to human adenoviruses were kindly supplied by Professors R. Wigand (GFR) and J. C. Hierholzer (USA). The set received from Wigand contained 31 rabbit type sera in the form of nine pools, i.e. 3—4 types pooled in each of the nine vials (Nos I—IX), while Hierholzer's material was a set of 34 monospecific equine type sera, each freeze-dried separately. The latter set was complete except for lack of a type 30 serum, while among the rabbit type serum pools the human adenovirus types 32, 33, 34 and 35 were not represented. On Wigand's recommendations, we used the serum pools in undiluted form in the neutralization tests. The monospecific horse sera were diluted before use.

Virus. The maintenance medium of primary rabbit kidney cell cultures of the rabbit adenovirus strain RA-1 was used as virus. The titre of the virus-containing medium was determined as 10^5 $TCID_{50}$/0.2 ml from the presence of intranuclear inclusions in stained preparations.

Cell culture. Primary rabbit kidney epithelial cell cultures were used throughout. The rabbits used as kidney donors were 3—7 days old. Hanks' balanced salt solution, containing 10% calf serum was used as growth medium, with streptomycin and kanamycin added as antibiotics. Plain Hanks' solution with antibiotic served as maintenance medium. The cultures were set up in Leighton tubes.

Neutralization tests. 1000 $TCID_{50}$ of the rabbit adenovirus strain RA-1 was used as standard virus dilution. The rabbit immune serum pools were used undiluted throughout. The equine sera were used in undiluted, and 10, 10^2 and 10^3 times diluted form. A mixture of equal volumes (0.5 + 0.5 ml) of virus and serum was incubated at 37 °C for 1 h. After incubation, 0.2 ml of the mixture was inoculated into primary rabbit kidney cell culture, following exchange of the growth medium for maintenance medium. The strain RA-1 was simultaneously titrated in cell culture. A series of non-inoculated cell cultures was set up as control. For evaluation, the coverslip cultures extracted from the Leighton tubes were stained with acridine orange. Determination of the neutralization titre was based on the presence or absence of intranuclear inclusions.

Immunoabsorption test. Seven of the nine rabbit immune serum pools received from WIGAND were found to contain antibodies to the rabbit adenovirus RA-1. Of these, one pool (No. VIII) was used for the immunoabsorption test as follows: the suspension of the rabbit adenovirus RA-1 was sedimented by ultracentrifugation (Beckmann type L2-65B; rotor SW 27) at 56 000 *g* for 2 hrs. To the pellet, 2 ml pool No. VIII was added, and after thorough shaking the system was incubated for 1 h at 37 °C and ultracentrifuged again. The supernatant was used as absorbed immune serum.

Neutralization test with the immunoabsorbed pool VIII and the specific adenovirus types (types 17, 22, 23 and 24). A sample of the immunoabsorbed pool VIII was freeze-dried and sent to the Adenovirus Reference Centre (Homburg, GFR) for testing for virus neutralizing effect on the specific human adenovirus types 17, 22, 23 and 24, in HeLa cell cultures.

Results

Seven of the nine rabbit immune serum pools received from WIGAND did neutralize the rabbit adenovirus strain RA-1, while the remaining two (Nos III and VII) did not.

The immunoabsorbed pool No. VIII failed to neutralize the rabbit adenovirus RA-1 in primary rabbit kidney cell culture, and the specific human adenovirus types (17, 22, 23 and 24) used for its preparation in HeLa cell culture, as it was shown in this laboratory and in the Adenovirus Reference Centre in Homburg, respectively.

Treatment of primary rabbit kidney cell cultures with the immunoabsorbed pool No. VIII in itself was followed by appearance of intranuclear inclusions up to a serum dilution of 1 : 10, indicating that excessive RA-1 virus persisted in the pool after absorption.

The presence of excessive RA-1 virus did not, however, interfere with the neutralization tests performed in HeLa cell cultures, because the genuine adenovirus fails to propagate in heterologous tissue culture.

All horse type sera to human adenoviruses received from Dr. HIERHOLZER failed to neutralize the genuine rabbit adenovirus. It should be noted that part of these sera proved to be cytotoxic in an undiluted state.

Discussion

The experimental studies reported in this paper have unequivocally shown that the virus strain RA-1, isolated in this laboratory from rabbits in 1975, is a genuine rabbit adenovirus rather than a human or animal adenovirus carried by rabbit (BODON et al., 1979; PROHÁSZKA and BODON, 1980). The failure of horse type sera to human adenoviruses (types 1—29 and 31—35) to neutralize the RA-1 virus has been regarded as a firm proof of the latter's genuineness.

We were, however, surprised to find that seven of the nine human adenovirus type serum pools available did neutralize the applied 1000 $TCID_{50}$ of the genuine RA-1 virus when used in an undiluted state.

Attempts at immunoabsorption of pool No. VIII with the ultracentrifugal pellet of the rabbit adenovirus were successful, as substantiated by the negative result of the neutralization test. The failure of the immunoabsorbed pool VIII to neutralize the specific human adenovirus types used for its preparation signified that not only the antibodies to the rabbit adenovirus, but also those to the human adenoviruses were removed by immunoabsorption. A possible explanation was that — as we learned later — the pools had originally been prepared from diluted immune sera. Further dilution during immunoabsorption obviously accounted for disappearance of the neutralizing activity.

A further proof of the genuineness of the RA-1 strain is the fact that none of the monospecific equine antisera to human adenoviruses contained neutralizing antibodies to the rabbit virus. It should be noted that the greater part (60%) of the monospecific sera proved to be cytotoxic for the primary rabbit kidney cell cultures when used in the undiluted state.

Other authors (LANGE et al., 1968) also reported detection by immunofluorescence of intranuclear virus synthesis in HeLa cells infected with types 2, 5 and 12 of human adenovirus. LANGE and his colleagues used sera of clinically healthy rabbits in the control series, expecting these to be free from adenovirus antibodies, but they were surprised to find a weak fluorescence also in the tissue cultures treated with the "clean" sera. The cited authors explained the phenomenon by postulating a latent adenovirus infection among the clinically healthy donors, but we believe that they saw a group-specific reaction.

It follows that consideration should be given to the possible presence of antibodies to genuine rabbit adenovirus in all rabbit serum tests in which the presence of these might interfere with the results.

Summary

Serological evidence has been presented that the adenovirus strain RA-1 isolated in this Institute from rabbits for the first time in the world is a genuine rabbit adenovirus rather than some known human or animal adenovirus transmitted by infection. The rabbit adenovirus was not neutralized by any of 34 type-specific horse antisera to human adenoviruses, but was neutralized by certain pools of rabbit immune sera to human adenoviruses. This signified the presence of antibodies to the rabbit adenovirus in the rabbit type sera, due to previous infection of donor rabbits by the genuine rabbit adenovirus. Since the antibodies to the rabbit adenovirus are capable of a group-specific reaction, their possible presence should be taken into consideration in all adenovirus tests performed with antisera of rabbit origin.

After immunoabsorption with the RA-1 strain, the rabbit immune serum pool No. VIII failed to neutralize not only the genuine rabbit adenovirus, but also the specific human adenovirus types (types 17, 22, 23 and 24) used for its preparation. The reason was that the type-specific sera had been diluted before pooling, and further dilution during immunoabsorption accounted for disappearance of their neutralizing activity.

None of the equine antisera to human adenoviruses contained antibodies to the RA-1 strain. It follows that the neutralizing effect of certain rabbit immune serum pools on the RA-1 strain was due to the presence of specific antibodies to RA-1 in the sera, i.e. to previous infection of donor rabbits by the genuine rabbit adenovirus.

Acknowledgements

The authors are indebted to Professor R. WIGAND (Adenovirus Reference Centre, Homburg, GFR), for supplying the rabbit immune serum pools used in this study, for neutralization tests of the immunoabsorbed pool VIII, and last but not least for valuable advice. Thanks are also due to Dr. J. C. HIERHOLZER for making available the set of equine type sera to human adenoviruses. The excellent technical assistance of Mrs. V. VASS and Mr. S. OROSZ is greatly appreciated.

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INTERRELATIONSHIP BETWEEN THE TOTAL PROTEIN CONTENT OF BOVINE SEMINAL PLASMA AND BEHAVIOUR OF THE SPERMATOZOA AFTER FREEZING-AND-THAWING

By

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The seminal plasma of mammals plays a decisive role in the protection of spermatozoa during cooling and freezing, and against cold shock.

The protein secretions of the accessory sexual glands form in many species a membrane-like coating on the surface of the sperm cells released from the epididymis (TAUBER et al., 1975; MATOUSEK, 1964). Proteins may increase the permeability of biological membranes, and it has been suggested that the basic proteins of the seminal vesicle have a similar effect on the membrane of spermatozoa (HIBBITT and BENIANS, 1971). High protein content in boar semen was shown to render the spermatozoic cell membrane susceptible to injury by cooling and deep-freezing (LAVON and BOURNSELL, 1971). Certain investigators believe that the seminal plasma proteins bind to the external membrane (plasma proteins and acrosome) of the spermatozoa (MOORE and HIBBIT, 1976). It is widely accepted that the membrane, viability, motility, survival and storage properties of spermatozoa are influenced by seminal plasma proteins (DOTT, 1961; WALES and WHITE, 1963; HIBBIT and BENIANS, 1971; MOORE and HIBBIT, 1976; LINDHOLMER et al., 1974).

This paper is a report of experimental studies on the effect of the protein concentration of bovine seminal plasma on the quality of bull semen, and on the storage life and fertility of the spermatozoa.

Materials and methods

Sixty-nine A.I. sires were selected for the study from the stock of the Artificial Insemination Centre, Budapest. Semen production and other reproduction-biological parameters of the bulls were followed up for 10 weeks. Samples of semen were taken every fifth day, and were examined as usual. The bulls were divided into three groups by semen quality as follows.

Group I — 25 bulls: Donors of excellent semen*t* with good freezing properties. These served as controls.

Group II — 22 bulls: donors of good semen which, however, showed quality deterioration after deep freezing for 9 min at —196 °C.

Group III — 22 bulls: donors of semen of poor quality.

Group III — 22 bulls: donors of semen of poor quality, and as such ab ovo unsuitable for deep-freezing.

The sires were healthy, and were kept under a regular veterinary supervision. The conditions of feeding and management were good. The examinations were performed during the months October, November and December in 1976.

Semen samples were collected in artificial vagina, and were centrifuged immediately at 3000 r.p.m. The seminal plasma was withdrawn with a glass pipette. Blood samples were withdrawn from the jugular vein after semen sampling. The serum and semen samples were equally stored at —20 °C until use.

Before use, the seminal plasma samples were diluted 20 times, the serum samples 10 times in saline. The protein content was determined with an autoanalyser.

Results

The mean protein contents of seminal plasma, with standard deviations, were assessed for the three groups as 5.8 ± 0.75, 7.6 ± 1.2 and 8.02 ± 1.01 mg/100 ml, respectively. The corresponding mean values of serum total protein were 7.9 ± 1.17, 7.27 ± 1.3 and 9.16 ± 1.9 mg/100 ml (Table I).

Table I

Protein concentrations in the seminal plasma and blood serum of A.I. bulls classified into three groups by semen quality

Protein content, mg/100 ml	Groups		
	I	II	III
	mean ± SD		
Seminal plasma	5.8±0.7	7.6 ±1.1	8.0 ±1.0
Blood serum	7.9±1.1	7.27±1.2	9.16±1.9

* Means determined from measurements conducted over 10 consecutive weeks.

Table II

Inter-group differences in seminal and serum protein concentrations as determined by analysis of variance

Groups compared	Degree of freedom	Significance
I×II seminal plasma	45	P<0.001
serum	42	N.S.
I×III seminal plasma	42	P<0.001
serum	46	P<0.05
II×III seminal plasma	45	N.S.
serum	36	P<0.001

N.S., not significant; P<0.001 significant.

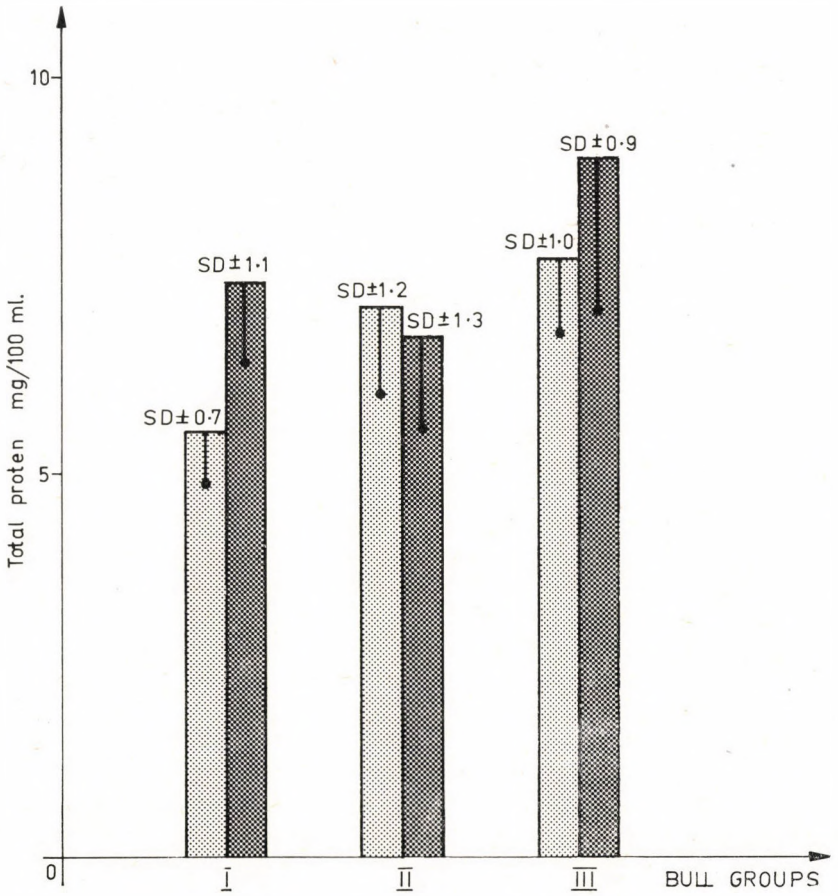

Fig. 1. Total protein concentrations in seminal plasma and blood serum of A.I. bulls classified into three groups by semen quality. ▦ seminal plasma; ▩ blood serum. The inter-group difference is significant ($P<0.001$) for group I *vs.* II and group I *vs.* III, not significant ($P<0.05$ for the seminal plasma but significant) ($P<0.001$) for the serum between groups II—III, and not significant for either serum or plasma between groups I—II and I—III

The difference in seminal plasma protein concentration was highly significant for group I *vs.* II group I *vs.* III, but not the difference for group II *vs.* III was significant (Table II).

The difference the total serum protein concentrations was highly significant between groups II and III, less significant between groups I and III, and not significant between groups I and II, as assessed by variance analysis.

The mean numbers of the spermatozoa exhibiting progressive motility and the mean germ cell concentration showed a highly significant negative correlation with the total seminal plasma protein content in both fresh and

Table III

Semen quality parameters* in the three groups of A.I. sires studied

Group	No. of bulls	pH	Volume, ml	Germ cell conc. $\times 10^9$/ml	Motility, %	Motility after freezing and thawing
I Excellent	25	6.4	5.7	1.4	71	50
II Quality deterioration by freezing	22	6.4	5.5	1.1	61	21.5
III Poor (not freezable)	22	6.5	5.2	0.95	34	12

* Mean values calculated from measurements over 10 consecutive weeks.

Table IV

Intergroup differences in semen parameters as assessed by analysis of variance

Groups compared	pH	Volume, ml	Germ cell conc. $\times 10^9$/ml	Motility, %	Motility after freezing-and-thawing, %
I× II	N.S.	N.S.	$P<0.001$	$P<0.001$	$P<0.001$
I×III	N.S.	N.S.	$P<0.001$	$P<0.001$	$P<0.001$
II×III	N.S.	N.S.	N.S.	$P<0.001$	$P<0.001$

N.S., not significant; $P<0.01$ significant; $P<0.001$ highly significant.

frozen-and-thawed semen samples (Table I). The correlation of the quantity of total seminal plasma protein with the other tested parameters was not significant (Table IV).

Discussion

Studies of mammalian seminal fluids have suggested that certain essential substances become lost in consequence of semen dilution (BLACKSHAW, 1953). Such losses can be partially prevented by addition of K^+ or certain large-molecular compounds to the semen or diluent (CHANG, 1959). In the diluted canine semen the viability of the germ cells depends on the presence of proteins capable of averting the untoward effects of dilution. MORITO and CHANG (1971) observed that addition of protein to the diluent was favourable for the preservation of germ cell motility.

The experimental observations reported in this paper substantiate LINDHOLMER's (1974) finding that in human seminal plasma the number of spermatozoa exhibiting progressive motility showed a negative correlation with the total protein concentration. LAVON and BOURSSNELL (1971) has postulated that membrane lipoproteins were responsible for the changes tak-

ing place in the quantity, composition, and electrophoretic properties of proteins during the maturation of bull spermatozoa. We agree with RYSER and HANCOCK (1965); REW and MCLAREN (1970b) and HIBBITT and BENIANS (1971) that proteins may increase the permeability of biological membranes and that this may also be the case with the proteins secreted in the seminal vesicle and the germ cell membrane. LAVON and BOURNSELL (1971) demonstrated in studies of boar plasma that the high concentration of proteins originating from the seminal vesicle rendered the germ cell membrane sensitive to the noxious effects of cooling and deep-freezing. MOORE and HIBBITT (1976) have postulated that the external membrane of the spermatozoa serves as binding site for proteins originating from the seminal plasma. This accords well with the suggestion of BOURNSELL and COOMS (1966) that adsorption of the seminal proteins by the spermatozoan membrane presupposes that the latter acquires in the epididymis a negative charge (YANNGIMACHI et al., 1972), which attracts positively charged proteins. Many authors have observed a noxious effect of the seminal plasma on the spermatozoa of various mammals during cooling and cold shock. FULKA et al. (1965) reported that treatment of preserved bull spermatozoa with secretion from the seminal vesicle rendered the germ cells sensitive to cold shock. Our own findings substantiate the observation of MOORE and HIBBITT (1976) that the seminal proteins become attached to the membrane of spermatozoon, and affect it adversely during cooling. This could account for disruption of the germ cell membrane under the protein-enhanced cold stress, and for the consequent loss of motility and of resistance to deep-freezing. This is, of course, only a hypothesis; the full understanding of the biological role of seminal plasma proteins requires further studies along this line.

Summary

The protein contents of seminal plasma and blood serum of A.I. sires were determined with the auto-analyser technique and were correlated with certain parameters of serum quality.

The A.I. sires included in the test were graded on the basis of semen quality, such as (1) excellent, (2) originally good, but deteriorated by deep freezing, and (3) poor and *ab ovo* unsuitable for preservation. The mean protein contents for seminal plasma were assessed in the three categories as 5.8 ± 0.7, 7.6 ± 1.2, $8.2. \pm 1.01$, and those for the serum as 7.9 ± 1.17, 7.27 ± 1.3, and 9.16 ± 1.9 mg/100 ml, respectively.

The concentration of spermatozoa, and the proportion of those exhibiting a progressive motility showed a negative correlation with the protein contents of the seminal plasma and serum both before and after deep-freezing. A high protein content of the seminal plasma may cause an increase in the permeability of the germ cell membrane and thus promote cold shock and injury of the cells during cooling and freezing.

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EFFECT OF ZINC SULPHATE SUPPLEMENTATION ON AMINO ACID ABSORPTION FROM PIG INTESTINE.

I. INTESTINAL LOOP TESTS

By

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(Received June 7, 1979)

The performance of high-production breeds greatly depends on appropriate feeding. Under the large production systems the dietary needs of the animals has differed to an ever increasing degree from those shown in traditional conditions of husbandry. The formulation of adequate feeds presupposes the precise knowledge of the altered requirements and of the factors influencing feed utilization.

The present studies were centered on the interaction of trace elements and amino acids during intestinal absorption. First, different dietary doses of zinc were examined for influence on amino acid absorption. This investigation was prompted by recent observations on interrelations between zinc and protein absorption.

Certain amino acids (*e.g.* histidine) were found to promote (WEITZEL et al., 1957), others (*e.g.* arginine; LEASE, 1975) to inhibit, the intestinal absorption of zinc. Also, oligopeptides were found to stimulate (NIELSEN et al., 1966), while peptides extracted from soybean, pea, rapeseed and mustard seed to depress, zinc utilization through forming highly stable complexes (LEASE, 1967). In the intestine of the dog HAHN and EVANS (1973) demonstrated a zinc absorption promoting oligopeptide of pancreatic origin, with presumably free metal binding sites.

According to present knowledge, proteins pass from the intestinal lumen into the blood usually in the form of oligopeptides, less often in the form of free amino acids (MANCHESTER, 1976). Presumably zinc, too, plays a role in the absorption of the latter.

Materials and methods

Scheme of the experiment

Eighteen castrated white bacon pigs, 25—35 kg in weight, were used in a total of 37 experiments.

The animals were fed ad libitum throughout the experiment. The components and main constituents of the diet were the following: corn 51%, barley

6.7%, wheat 15%, fat 5.0%, soybean extract (crude protein 45%) 10.0%, fish meal (crude protein 70%) 5.0%, alfalfa meal 4.5%, AP-17 1.0%, lime 1.0%, salt 0.3%, piglet premix 0.5%; metabolizable energy Kcal/kg 3150, crude protein 16.1%, calcium 0.9%, phosphorus 0.6%, iron 150 ppm, zinc 100 ppm, copper 30 ppm, iodine 0.5 ppm.

For intestinal absorption studies, Thiry-Vella-type isolated intestinal loop preparations were obtained as described by KOVÁCH (1954). In pigs under anaesthesia, the ileum was transsected about 5 cm away from the caecum and 20 cm away from the stump in oral direction. The mesentery was left intact. The excised segment was bent to a loop, lifted from the abdomen at both ends, and fixed with sutures to peritoneum and skin. Lateral anastomoses were formed to maintain communication.

To determine the site of absorption of the amino acids, in some cases a Thiry-Vella-type fistula was also prepared from the anterior third of the jejunum, exactly as described above. To promote wound healing, a cow's milk and potato diet was fed to the operated animals for 4 days.

Eight days after surgery, the isolated intestinal loop was rinsed with distilled water. The first absorption experiment was performed 10 days after healing of the surgical wound.

Experimental and control tests were set up in the same isolated loop, in a predetermined sequence. First the absorption of a solution of polyethylene glycol (PEG-4000) was observed, then, 48—72 later, solutions containing amino acids, PEG-4000, and zinc in rising amounts, were applied.

The tests were always performed in the same period of the day (between 9 and 10 a.m.), after fasting for 14 hrs. The animals were restrained in a Burdenjuk-type crush, transformable to an operating table (B. KOVÁCS, 1977), and the fistula openings were prepared for closure. Twenty or 70 ml test solution, warmed to body temperature, was administered into the isolated loop. Ten and 30 min later, respectively, samples of intestinal content were taken for chemical tests, then the loop was rinsed with distilled water. The rinsing fluid was centrifuged and passed through a G-5 glass filter. The filtrate was used for concentration measurements.

Test materials

For absorption studies, an aqueous solution containing amino acids and PEG-4000 was prepared. The solution contained 2000 ppm each of L-aspartic acid, glycine, L-methionine, L-isoleucine, L-lysine, L-histidine and L-arginine). PEG-4000 was dissolved at the level of 3000 ppm. As PEG is not absorbed from the intestine, its concentration increase is conclusive of the degree of water absorption (MALAWER and DON POWEL, 1967). PEG-4000 was, therefore, used to assess the absorption baseline.

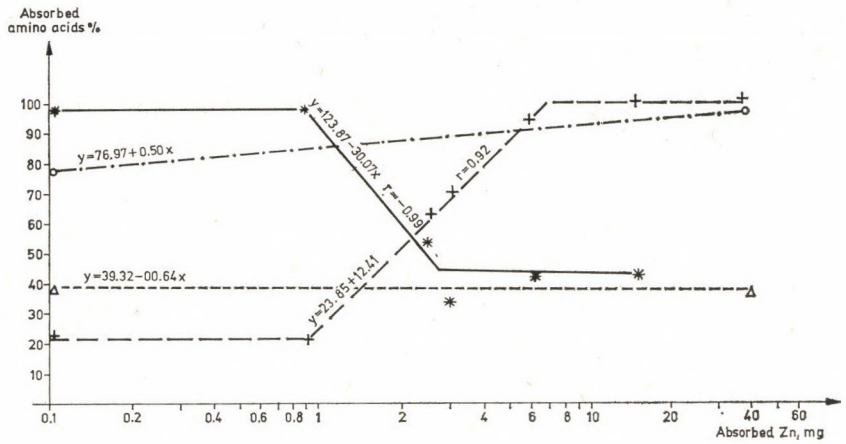

Fig. 1. The interaction between the absorption of aspartic acid, glycine, methionine, arginine and zinc. —·— aspartic acid; — — — glycine; - - - - - methionine; ——— arginine

In further tests, zinc was added to the amino acid + PEG-4000 solution at the concentrations of 50, 150, 225 or 1800 ppm, in the form of zinc sulphate ($ZnSO_4 \cdot 7\ H_2O$).

Determination of PEG and amino acid concentrations

Statistical evaluation. The concentration of PEG-4000 was determined by nephelometry, in samples diluted, de-proteinized with $BaSO_4$, and treated with gum arabic or $BaCl_2$-containing trichloroacetic acid. Turbidity was read one hour later at 650 nm against distilled water as blank.

Amino acid concentrations were determined with an automatic amino acid analyser. The two-column procedure was employed, and taurin was used as internal reference standard. The test material was applied in 0.5 μM expected quantity. The amino acid concentrations were calculated under consideration of the water absorption from the lumen.

Mathematical-statistical evaluation was made with the one-sample t test. Mean and standard error were determined. The correlation found, its significance and the linear regression coefficient are shown in Fig. 1.

Results

Absorption of amino acids from the two intestinal loops

First the intestinal segment most suitable for follow-up of the absorption and interaction of amino acids and zinc was identified by experimental approach. As opinions have been divergent on this aspect, we set up preliminary absorption studies simultaneously in the jejunum (anterior loop) and in the

ileum (posterior loop). A solution containing PEG-4000, the above amino acids and 50 ppm zinc was used for this purpose. The absorption were expressed in per cent of the input amino acid. (The pre-experimental dietary protein and zinc supplies of the pigs corresponded to the standard requirements) (Natl. Res. Council, 1968; MÉSZÁROS, 1973).

It was found that all amino acids tested were absorbed at a higher percentage from the ileum than jejunum (although per cent absorption is dependent on the amino acid).

Absorption of amino acids in the presence of different zinc ($ZnSO_4 \cdot 7\ H_2O$) concentrations

The influence of different zinc concentrations on amino acid absorption was tested in the posterior (ileal) loop throughout. The results are shown in Table II (headings 1—3).

A considerable proportion of the amino acids became absorbed even in the absence of zinc. During a 30-min period, the greatest proportion was absorbed from arginine, the lowest from aspartic acid and lysine, while histidine, isoleucine and methionine were intermediate between the two extremes.

Addition of a small amount of zinc sulphate (50 ppm zinc) to the amino acid solution gave rise to a measurably greater absorption of all amino acids except histidine. Again, the greatest proportion was absorbed from the arginine, the further order being lysine, methionine, aspartic acid, glycine and histidine.

When the zinc concentration was raised to 150 ppm aspartic acid and glycine absorption still showed a rising tendency, but compared to the values obtained in the presence of 50 ppm zinc, no significant increase was found. From lysine 41% became absorbed, methionine and arginine absorption did not change, and histidine absorption was not followed at this level of zinc.

Extremely high zinc concentrations, four times (225 ppm) and about thirty times (1800 ppm) as great as the actual requirement, were also tested for influence on amino acid absorption. Samples were taken from the intestinal loop 10 min after application (headings 4—5, Table II). The absorption of the aspartic acids of acid character, and glycine, was promoted, that of the basic amino acids lysine and arginine was depressed, and the neutral amino acids were irresponsive to zinc. However, owing to individual variations, methodical errors and indirect assessment of the amino acid concentrations, the standard error was too great to permit an analysis for significance. Individual variation can be eliminated if the phenomena are pursued by repeated tests on the same animal. Fig. 1 shows the conditions of aspartic acid, glycine, methionine, arginine and zinc absorption in pig No. 7/1 (absorption time 10 min; volume of solution 20 ml).

Table I

Percentual absorption of amino acids from ileal and jejunal loop preparations

Intestinal segment (loop)	Amino acids					
	L-aspartic acid	glycine	L-methionine	L-lysine	L-histidine	L-arginine
jejunum (n=2)	28.8	58.9	61.2	49.1	18.5	92.6
ileum (n=2)	60.4	66.4	70.4	70.3	49.7	98.5

Table II

Percentual absorption of amino acids in the presence of different zinc concentrations

Group	Zinc level	Sampling time (min)	Amino acids						
			L-aspartic acid	glycine	L-methionine	L-izoleucine	L-lizine	L-histidine	L-arginine
1.	No Zn (n=6)	30	24.3±9.2	33.8±9.9	45.0±9.7	57.1±5.3	21.6±4.5	58.6	83.0±16.1
2.	50 ppm	30	55.3±15.2	55.6±11.7	61.3±13.4	—	62.2±9.6	49.9±20.2	98.5
3.	150 ppm (n=3)	30	61.2±18.9	66.4±14.6	58.7	—	40.8±15.6	—	99.2
4.	225 ppm (n=9)	10	67.6±10.6	64.4±12.6	53.7±11.6	60.6±14.0	50.6±10.5	—	74.5±7.7
5.	1800 ppm (n=4)	10	78.5±8.9	85.4±7.3	46.5±7.5	54.9±14.9	48.6±4.06	—	68.9±10.8
Significant difference between groups 1 and 2:			$p<0.05$	$p<0.01$	$p>0.05$		$p<0.05$		

Absorption of a small amount (0.9 mg) of zinc had no effect on the intestinal absorption of amino acids, but an increase of the zinc absorption to 2.5—6.0 mg accounted for increase of glycine absorption from the 21% baseline to the maximum, and depression of arginine absorption from 100% to 44%. Aspartic acid absorption also tended to increase, but the correlation was not significant owing to the high standard deviation. Methionine absorption showed no change.

Of 70 ml fluid 60—97% (mean: 82.7 ± 3.6) was absorbed in 30 min, of 20 ml fluid 15—40% (mean: 25.8 ± 2.4) was absorbed in 10 min. The quantities of absorbed amino acid and absorbed water showed no correlation.

Discussion

Identification of the sites of intestinal absorption can disclose important information on interactions. The bulk of zinc becomes absorbed from the small intestine, but literary data on the segment serving as primary site of absorption in the different species have been divergent.

In poultry, the ileum (KASARKIS and HOEKSTRA, 1974), in man and rat the duodenum (WALKER et al., 1973; EMES and ARTHUR, 1975; METHFESSEL and SPENCER, 1973) and in the pig the jejunum (DAHMER et al., 1972) has been described as the primary site of zinc absorption, while in the calf, absorption did not seem to differ between segments of the small intestine (HAMPTON et al., 1976).

Observation on amino acid absorption also are equivocal. Most authors agree that in man it is completed over the first 50—100 cm long portion of the jejunum (BORGSTROM et al., 1957), or at least until the ileum (SPENCER, 1960), while FROEHLICH (1972) found it to be similar in jejunum, ileum and duodenum. L-methionine was absorbed at a higher rate from the proximal than the distal small intestine of man (SCHEDL et al., 1968), while in rats the rate of absorption tended to increase distally (CRAMPTON et al., 1973). The bulk of alanine was absorbed from the jejunum, while the absorption of L-tryptophan, L-tyrosine and L-phenylalanine took place chiefly in the middle segments of the small intestine (SHEEHY and FLOGH, 1964).

In our experimental model, amino acid absorption was greatest in the ileum during the 30-min period of observation. About twice as much aspartic acid, lysine and histidine was absorbed from the ileal segment as from the jejunal one, while arginine became nearly completely absorbed from both loops. It was found that simultaneously-administered arginine may interfere with the absorption of histidine. According to LEASE (1975), arginine interferes with zinc absorption probably *via* inhibition of the absorption of the zinc-binding histidine.

Based on their absorption from both intestinal loops, amino acids could be ranked. Arginine and methionine were absorbed at the highest rate, and aspartic acid at the lowest, while the absorption rate of lysine and glycine was intermediate between the two extremes. This accords well with the absorption rates established by GITLER (1964) in equimolar amino acid pools.

Absorption of certain amino acids (aspartic acid, glycine, lysine) from the ileal loop was enhanced by the addition of $ZnSO_4$ (50 ppm zinc), while methionine absorption did not increase significantly, and histidine absorption remained practically unchanged in the presence of zinc.

The absorption rates of the tested amino acids differed considerably in the absence of zinc, but addition of a minimal quantity of zinc (50 ppm) accounted for a certain equalization, which obviously favours protein synthesis. It follows that amino acid imbalance may also contribute to the depression of protein synthesis in zinc-depleted animals.

The zinc concentration accounting for optimum amino acid absorption in the present study proved to be identical with the physiological zinc content of animal tissues (40—80 ppm; ANON., 1973), to which the zinc supplementation of premixes (at least 50 ppm; MÉSZÁROS, 1973) has been adjusted.

Addition of 150 ppm zinc to the amino acid solution altered the conditions of absorption to a lesser degree. Arginine was easily absorbed even in the absence of zinc, but addition of a minimal amount of $ZnSO_4$ (50 ppm zinc) accounted for its nearly complete absorption. Further elevation of the zinc sulphate content depressed the absorption of basic amino acids and enhanced that of aspartic acid and glycine, but neither effect was significant statistically.

The main tendencies of zinc influence on amino acid absorption become expressed also at extremely high concentrations of zinc, and classification by absorption properties (neutral amino acids; glycine; acid and alkaline amino acids) remained the same as established in classical absorption studies (WISEMAN, 1964).

For evaluation, it should be taken into consideration that amino acids may mutually interfere with the absorption of one another. Using an equimolar pool of amino acids (GITLER, 1964) has shown that the transportation of the low-affinity amino acids commenced only after a certain quantity of the easily absorbable ones had been absorbed, i.e. after the competitive inhibition had decreased. A competitive antagonism can be expected above all within groups of amino acids (e.g. between arginine, lysine and histidine). A separate transport of different amino acids (neutral, proline, glycine and alkaline) was observed in most animal species (WISEMAN, 1964).

Amino acid absorption was generally poorer from equimolar pools than from albumin hydrolysates (GITLER, 1964). This seems to indicate an interrelation between the quantitative supply and absorption of amino acids.

In the sequence established by DELHUMEAU (1962) on the basis of absorption rates from rat intestine, the first 11 amino acids were essential or semi-essential, while the subsequent ones were non-essential. It should also be taken into consideration that the given amino acid composition of the diet or pool, and the actual needs of the animal may probably also influence the rates of absorption. With equimolar pools, the quantitative relations of essential and non-essential amino acids differ considerably from the actual needs; e.g. all amino acids of DELHUMEAU's sequence except lysine were present in excess. In this light, the sequence has been modified by giving the first place to lysine, while methionine, 50% in excess, has been referred to the fourth or fifth place, followed by histidine and arginine, present in 60 and 80% excess, respectively. The non-essential amino acids also were present, more or less, in excess. Under the National Research Council Recommendations (NRC-NAS, 1968), a 14% dietary crude protein supply has been prescribed for the growing pig. Our experimental animals received 16% in their diet. The limiting order of amino acids of the experimental diet was the following: methionine, lysine (lysine deficiency may be enhanced by arginine excess, owing to arginine—lysine antagonism) (D'MELLO and LEWIS, 1970; KADIRVEL et al., 1974); threonine, tryptophan, isoleucine; valine, histidine, phenylalanine; leucine, arginine, all in considerable excess.

In the pool administered into the intestinal loop, as limiting amino acid lysine was the first in the order, relative to which methionine was present in 30% excess, and histidine and arginine in 70—75% excess.

Since the absorption sequence observed in the isolated loops (Arg-Met-Lys-Gly) differed from the limiting order of amino acids established in the diet and experimental pool, and from DELHUMEAU's sequence as well, there was no indication of any correlation between the limiting amino acid sequence and absorption intensity. It would be of interest to investigate the absorption effects of amino acid nutrition at different levels of amino acid supplementation.

The mean absorption rates (Table II) and their interrelations (Fig. 2) permit different explanations of the importance of zinc. It might be supposed that zinc ions play a special role in amino acid transport, due to an increase or decrease in transport enzyme activities in their presence. The dissimilar absorption rate of certain amino acids in the presence and absence of zinc may contribute new aspects to the understanding of the traditional classification of amino acids. An other possible explanation is that shift of the pH — from 6.8 to 4.5 — on addition of more sulphate to the amino acid-zinc sulphate mixture may have dissimilarly affected the different groups of amino acids. Let us refer in this context to CHRISTENSEN's et al. (1973) speculation that the H^+ gradient may influence the transport of basic amino acids. CSÁKY (1969) demonstrated in sugar absorption studies that rise of the

H^+ concentration accounted for a gradual inhibition of the sodium pump. In this condition, the different amino acid groups may become absorbed at different sites during their forward passage in the intestinal tract, as suggested by us earlier.

Another aspect to be taken into consideration is that absorption experiments with free amino acids are not conclusive of the conditions of peptide absorption. It is known that amino acids and peptides become absorbed independently of one another, by different mechanisms (MANJUSTI DAS and RADHAKRISNAN, 1976). Zinc may nevertheless play a role in peptide absorption, since it either enhances (ALBANESE, 1974) or influences by absorption-related interactions SKORYNA and WALDRON-EDWARDS, 1971) otherwise the activity of many enzymes (dipeptidase, aminopeptidase, arginase, histidine-deaminase, carboxypeptidase, etc.). Splitting of the dipeptides takes place in the intestinal brush border, *i.e.* in the localization of the dipeptidases to which a carrier function has been attributed in conditions of the simplest transport model (MUNCK, 1976).

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Summary

The effect of zinc on amino acid absorption was studied in a total of 37 experiments on 18 white bacon pigs 25—35 kg in weight. Thiry-Vella-type intestinal loop preparations were obtained by isolation of the ileum and the middle part of the jejunum. An amino acid solution, containing 2000 ppm each of L-aspartic acid, glycine, L-methionine, L-isoleucine, L-lysine, L-histidine and L-arginine was administered into the isolated loop, with and without addition of $ZnSO_4 \cdot 7\ H_2O$, corresponding to 50, 150, 225 or 1800 ppm of zinc. The same loop preparation was generally used for establishment of the baseline and for testing the effect of rising zinc concentrations. The quantitative decrease of amino acids, zinc and water was determined in the loop content after absorption times of 10 and 30 min. All amino acids were absorbed in a greater percentage from the ileum than the jejunum, and the rate of absorption depended on the amino acid. The absorption of the amino acids was considerably enhanced, and the rates of absorption of different amino acids became practically equalized (with the exception of arginine) in the presence of a low concentration of zinc sulphate (50 ppm zinc). Repetition of the tests in the same loop preparation showed that raising of the zinc concentration accounted for an increase of glycine absorption rate and a decrease of arginine absorption, but it had no effect on the absorption rate of neutral amino acids.

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STUDIES OF THE SO-CALLED "PARTUS SYNDROME" OF DAIRY COWS

By

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The profitability of large dairy cattle units greatly depends on herd health control, on prolongation of the useful life span of cows and on elimination of the performance-limiting factors accounting for culling. The main cause of culling has been infertility due to peripartal metabolic disorders and udder diseases of various aetiology.

Herd health control has recently been widely based on blood profile studies. Seasonal fluctuation of certain blood parameters are (PAYNE et al., 1974); e.g. the blood urea nitrogen (BUN) and haemoglobin concentration are higher in summer than in winter (HEWETT, 1974; LANE and CAMPBELL, 1969; LITTLE et al., 1976; MERALL, 1976), owing to reduced dietary protein and energy supplies during the cold season (MANSTON et al., 1977). However, the albumin, Mg and Cu show little seasonal changes if any (PAYNE et al., 1974; ROSS and HALLIDAY, 1975; SOMMER, 1970). Checking of the blood profile around calving may be of prognostic value in respect of imminent puerperal and reproductive disorders (ARBEITER, 1973; BOGIN and SOMMER, 1978; BOSTEDT et al., 1977; GOODALE et al., 1978; HARASZTI et al., 1979; KING et al., 1976). The effects on re-fertilization of management and feeding during the dry period, and of calving, have widely been studied in cows of different breeds, ages, and performance standards. As peripartal disorders usually occur in association with symptoms related to calving, the clinical complex has been termed in the literature as "partus syndrome" (or "parturition disease"). This term refers, besides metabolic disorders due to faulty management and feeding during the dry period, to the consequences of the stress imposed upon the animal by advanced pregnancy, calving, and subsequent onset of lactation. The joint effect of these untoward influences leads to a latent or clinical metabolic dysfunction, retention of fetal membranes, puerperal uterine diseases and ovarian dysfunction and subinvolution. Thus the term "partus syndrome" also refers to the common pathogenetic factor(s) operative in the syndrome, since all changes involved in the latter have their origin in the structural and functional alterations of the liver during the dry period.

Investigations into the aetiological and prognostic aspects of the partus syndrome, based on herd studies, are reported in this paper.

Materials and methods

On a total 45 Holstein-Friesian and 104 Hungarian Red Pied cows were included in the study. The preceding annual milk outputs were 6500 and 3600—3800 litres, respectively. Both herds were adequately managed and fed, and no clinical disease occurred during the period of study.

In the Holstein-Friesian herd, changes of SGOT and of the differential leucocyte counts were followed for 8 days, each before and after the calvings, which took place from March to May 1978. In 17 cases, the serum glycocorticoid concentrations were additionally assessed for 14—15 days post partum, and in 18 cases the plasma progesterone level was determined by radioimmunoassay for about 42 days post partum to obtain information on ovarian function.

In the Hungarian Red Pied herd, changes of the serum cholesterol level were followed up from partus through 25 days.

For SGOT and cholesterol determination blood samples taken from the jugular vein every other day were examined by spectrophotometry (spectrophotometer MOM, Budapest), using the LA ROCHE diagnostic kit and the BOEHRINGER Biochemica Test, respectively. For leucocyte counting, the blood sample was diluted 20-fold in Türk solution, and a Buerker chamber was used. The plasma glycocorticoid levels were determined by a modified version of MURPHY's (1967) competitive protein-binding assay (CPBA).

To obtain more information on the adverse effects of confinement during the dry period, Holstein-Friesian cows put dry were walked daily over a distance of 2800—3000 m, from December 1, 1978, to May 31, 1979, *i.e.* for 6 months, where after losses due to spontaneous death, emergency slaughter and culling, as well as service period length and fertilization index were compared with similar values assessed during the 6-month dry period from Dec. 1, 1976 to May 31, 1977 in which the cows had not been exercised. The average annual milk yield was 6800 litre in this herd.

The following daily rations were fed during the period of observation, *i.e.* in weeks 6—8 of the dry period:

Group I — Maize silage, 20—25 kg
alfalfa hay, 3—4 kg
"dry cow B" formula, 6 kg

Group II — Maize silage, 16—18 k
alfalfa hay, 8 kg
"dry cow B" formula, 6 kg

Composition of the "dry cow B" formula:

3 kg dairy cow feed + 3 kg mixed grain fodder (bran, corn, barley).

The "dry cow B" formula contained 35% dry matter, its digestible crude protein to starch equivalent ratio was 1 : 5.7, and its Ca/P ratio was 1.168 : 1.

Results and discussion

The mean ($\bar{x} \pm S$) pre- and post-partal SGOT levels of the Holstein-Friesian cows are shown in Fig. 1.

The SGOT tended to rise from day 8—5 before calving and reached its peak between days 1 and 4 after it, conform to the earlier observation of BOSTEDT et al. (1977). The peripartal increase of the SGOT over the upper physiological limit seemed to be due, at least partly, to partus-related non-hepatic release of GOT and, accordingly, attained its maximum between 24 and 48 h post partum (BOSTEDT, 1974). GOT activity was found to be highest in the skeletal muscles (GERBER et al., 1973); according to newer findings, however, it is twice as high in the cow's liver as in its skeletal or heart muscle; bulls show four times as high GOT activity in the liver as in the myocardium (DÖCKE and WORCH, 1962). BOGIN and SOMMER (1976) also found maximal activity in the bovine liver. GLAWISCHNIG et al. (1971) reported rise of SGOT activity in CCl_4-poisoned cows, while the creatine-kinase activity remained unchanged. Hepatic functional disorder accounts for a considerable increase of the SGOT level. The direct or indirect hepatic influence of peripartal ruminal dysfunction and metabolic imbalance can easily precipitate this condition in the period around calving.

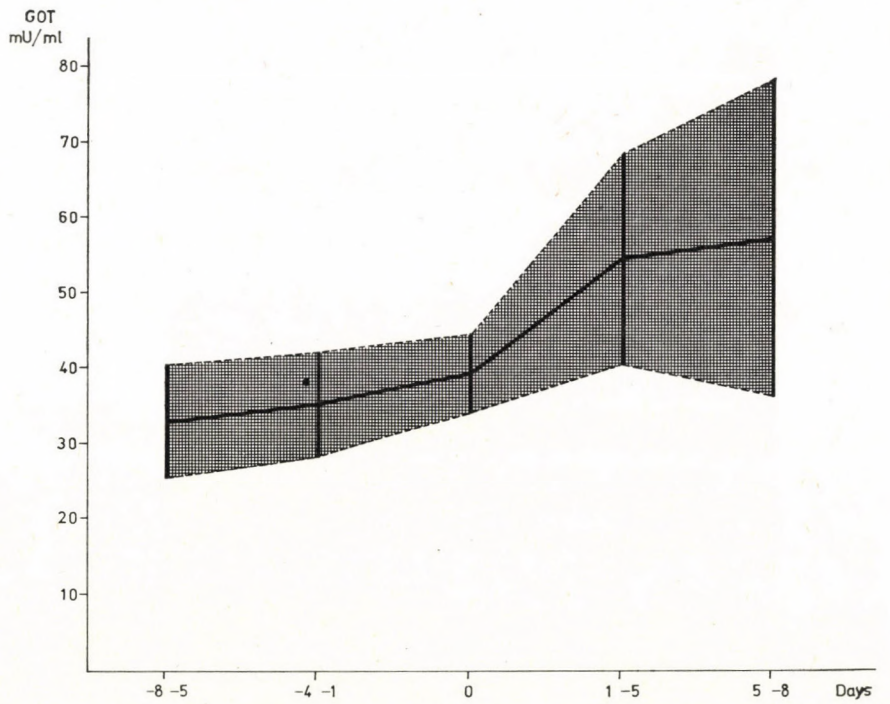

Fig. 1. Fluctuations ($\bar{x} \pm S$) of SGOT between day 8 ante partum and day 8 post partum

Determinations of serum GOT and cholesterol levels in 5 dry cows, all in excellent health, have unequivocally indicated the great diagnostic value of these two parameters. Despite their apparently good condition, all the five cows became clinically ill after calving. One of them had been recumbent from day 2 ante partum, and died immediately after calving. This animal had a SGOT level of 310 mU/ml on day 5 ante partum. All the five cows had shown a decrease of serum cholesterol as the SGOT was rising. It is of interest that two months after calving, *i.e.* long after the conclusion of our study, 2 of the remaining 4 cows had to be emergency-slaughtered on account of chronic inappetence and indigestion, and the other two were culled for uterine infertility due to retention of fetal membranes.

BOSTEDT (1974) observed that primiparous heifers showed a greater rise in SGOT after calving than 3—6 years old cows. In our experiments, cows aged more than 5 years showed a significant rise in SGOT and a significant drop of serum cholesterol during the first days post partum. Blood glucose and inorganic P levels also rose and fell, respectively, in a significant degree, compared to the values of similarly-managed and fed, but less than 5-year-old cows of the same population.

SOMMER (1970) observed in 3-year studies that cows having a serum cholesterol level of 75 mg/100 ml 8-6 weeks before calving showed a 3, 2, 3 and 5 times greater incidence of retention, mastitis, stillbirth and ovarian disorder, respectively, than those having normal serum cholesterol levels during the critical period. In our own study, the serum cholesterol levels measured in 104 Hungarian Red Pied cows for 21—25 days after calving were analysed in comparison to the length of the service period. To facilitate evaluation, the cows were divided into three groups with regard to service period lengths, and the average serum cholesterol concentrations found in Groups II and III were analysed for significance of difference from Group I, which was regarded as physiological (Table III). The cows of Group III were culled on account of chronic endometritis, and/or ovarian dysfunction, after failure of repeated therapeutic attempts.

The total serum cholesterol level changes during lactation (HENRICSON et al., 1977); it is higher in lactating than in dry cows. Puerperal drop of the serum cholesterol level, usually accompanied by drop of blood glucose and rise of SGOT, often signifies changes accounting for infertility consequent upon uterine infertility (LOTTHAMMER et al., 1971; SOMMER, 1970; SOMMER and MARX, 1969). In such cases, slow involution accounts for prolongation of the service period and, consequently, of the calving interval (HARASZTI et al. 1978, 1979).

Although gestation and calving, are physiological conditions, both impose a minor to major stress, depending on the age and individual sensitivity of the cow.

Table I

Pre- and postpartal serum GOT and cholesterol levels of cows found diseased post partum

SGOT, mU/ml Cholesterol, mg/100 ml	Ante partum							Post partum				Remark
	—15—13	—12	—10	—8	—5	—4—3	—2—1	Calving day	1—2	4—5	6	
I. GOT	36	43			310	280	275					died
Cholesterol	129.2	77.6			58.1	27.3	27					
II. GOT		26	39.5	31	31	47	149		145	180	220	culled
Cholesterol		108.6	117.2	81.2	70.5	81.8	62.5		64.5	23.2	36.7	
III. GOT	21		21			31	25		48	330		culled
Cholesterol	79.2		52.9			83.8	47.3		28.1	62.1		
IV. GOT							210	250	210	120	125	emergency-slaughtered
Cholesterol							73.2	50.2	37.5	61.2	40	
V. GOT		35		29	31				180	140	150	emergency-slaughtered
Cholesterol		94.8		116.6	72.9				77.4	64.2	42.2	

Table II

Peripartal changes of serum GOT, cholesterol, blood glucose, Ca, inorganic P and Mg in cows younger and older than 5 years

		—3—1	Calving day	1—3	4—6	7—10
GOT	□	34.1 ± 5.4	51.9 ±21.8	63 ±21.6	123.4 ±14.2	94.2 ±62.3
	○	31 ± 4.8	35.3 ± 4.5	44.6 ±14.1	42.2 ±10.3	45.8 ±13.0
			P<0.01	P<0.01	P<0.01	P<0.05
Serum Cholesterol	□	77.3 ±23.7	77.3 ±20.2	71.8 ±11.1	70.6 ±12.6	80.9 ±15.2
	○	87.4 ±15.9	78.1 ±12.1	78.5 ±11.8	95.9 ± 5.5	99.6 ±12.9
					P<0.001	P<0.01
Blood glucose	□	56.3 ±12.8	81.9 ±25.8	62.3 ±21.1	46.3 ±14.5	42.2 ±12.6
	○	67.4 ±10.6	65.3 ±13.1	52.3 ±17.5	47.5 ±13	48.5 ±10.1
		P<0.05	P<0.05			
Serum Ca	□	12.98± 1.77	10.64± 1.99	9.89± 0.6	11.98± 2.03	11.82± 2.17
	○	11.78± 1.9	11.44± 1.8	10.23± 0.7	10.9 ± 1.4	11.6 ± 1.7
Inorganic P	□	5.8 ± 0.5	3.4 ± 0.7	4.8 ± 1.0	4.9 ± 0.7	4.8 ± 1.1
	○	6.3 ± 0.8	5.9 ± 0.5	5.7 ± 1.6	5.0 ± 0.9	5.2 ± 0.4
		P<0.05	P<0.001	P<0.10		
Serum Mg	□	3.01± 0.58	2.87± 0.45	2.6 ± 0.61	2.25± 0.32	2.38± 0.87
	○	2.72± 0.34	3.05± 0.54	2.67± 0.4	2.54± 0.45	2.71± 0.39
Carotene	□	537±203	721±213	440±106	349±65	367± 138
	○	496±96	597±227	505±120	480±91	434±113

□ Cows older than 5 years; n = 15. ○ Cows younger than 5 years; n = 15.

Table III

Dynamics of the serum cholesterol level during 21 to 25 days after calving (n = 104)

Calving day (0-day)	Days post partum					Length of service period (days)
	0—5	6—10	11—15	16—20	21—25	
Group I n=58	81.2±16.3	85.3±20.1	105.2±21.7	128.2±31.3	139.2±32.1	71.6±19
Group II n=28	86±14.5	82.4±23.4	104.7±28.7	117.2±26.3	114.1±38.5 P<0.01	135.9±24.6
Group III n=18	82.9±10.9	70.8±13.8 P<0.01	68.6±12.9 P<0.001	87.3±29.8 P<0.001	108.2± 9.6 P<0.001	culled

The total and differential leucocyte counts become altered in the immediate prepartal period and, especially, during calving, in that the total count and the proportion of neutrophils tend to increase, while the percentages of eosinophils and lymphocytes tend to decrease. Cows and women have shown similar puerperal shifts of the eosinophils count (BOSTEDT and BERCHTOLD, 1968; FRIEDBERG, 1970; SEIDEL et al., 1973). Changes in the differential count, such as eosinopenia, neutrophilic leucocytosis, lymphopenia, as well as accompanying phenomena of the stress syndrome also in the bovine (DÖCKE and WORCH, 1962; FORENBACHER, 1967; KARG, 1955; WEINRICH, 1960; WILLER, 1961, 1963). Peripartal changes in the differential counts of 45 symptomless Holstein-Friesian cows are shown in Table IV.

During calving, the total leucocyte and neutrophil counts reached maxima while the eosinophil count fell to a minimum. Postpartal changes in serum glucocorticoid levels, assessed from blood samples taken every other or third day within 14—15 days after calving are shown in Table V.

The serum glucocorticoid level rose from its prepartal 5 ng/ml value to 16.7 ng/ml during calving, then it gradually returned to 5 ng/ml (BAIRDS and MATHER, 1970; CONVEY, 1974; HEITZMAN et al., 1970; SMITH et al., 1973).

The post-partal normalization of the cyclic ovarian function also takes place gradually. The period of complete "sexual convalescence" varies individually with age, performance standard, and course of the preceding calving. Most cows show signs of restored ovarian function within 4 weeks, having ovulation between days 20 and 30 post partum, without any indication of a corpus luteum between days 12 and 18 (KUDLÁČ, 1971). Other authors observed the first ovulation in cows with an undisturbed ovarian function 19.5 or 19.1 ± 1.7 days after calving; the second and third ovulation followed on days 44.4 ± 13.2 and 63.7 ± 10.1, respectively (GOODALE et al., 1978; KING et al., 1976). The plasma progesterone level, which was 3.0 ± 0.7 ng/ml on the least prepartal day, dropped to 0.6 ± 0.1 ng/ml during calving and persisted at that low level for 9 days. It was slightly higher on postpartal days 2 and 3 than on days 7, 8, 12, and 13, but it never rose to 1 ng/ml, and in no case did the fluctuations exceed 0.5 ng/ml (GOODALE et al., 1978). The time of the first ovulation shows considerable individual variation and depends on the existing conditions; its recognition is hampered by its usually symptomless course. This prompted us to follow up ovarian activity by plasma progesterone determinations in samples taken every third day during the first two weeks post partum and at weekly intervals in the subsequent 4 weeks.

If checked weekly in weeks 5, 6 and 7 after calving, the plasma progesterone level has reached or exceeded 7 nmol/litre (i.e. 2.1 ng/ml) on at least a single occasion, the cycle of the cow can be regarded as restored from day 29 to 49 post partum (BOYD and MUNRO, 1979). As supported by CHOI et al. (1977), the first oestrus manifesting in characteristic symptoms had been

Table IV

Dynamics of differential leucocyte count around calving (n = 45)

	—4—3	—2—1	0	1—2	3—4	5—6	7—8
Total leucocyte count	7355±1719	7718±1933	8043±2221	6565±1638	6547±1574	6994±1721	6371±1743
Lymphocytes, %	40.7±28.7	50.1±12.9	42.2±12.3	55.5±15.2	58.3±13.0	59.4±13.9	59.1±14.1
Neutrophyls, %	38.1±10.7	40.3±12.5	51.5±11.3	35.2±14.2	36.6±13.1	33.2±13.7	32.1±14.4
Eosinophils, %	8.7±7.4	7.2±4.9	3.9±3.3	6.8±6.9	6.4±4.9	5.5±4.6	5.9±4.7
Basophils, %	0.5	0.3	0.3	0.1	0.2	0.4	0.2
Monocytes, %	2.1±1.4	2.2±2.3	2.2±1.7	2.4±1.4	2.8±1.4	2.4±1.5	2.3±1.5

Table V

Fluctuations of the serum glucocorticoid (ng/ml) level for 14—15 days after calving

Calving day (0-day)	n = 17				
	Days post partum				
	0—1	3—4	6—8	10—12	14—15
	16.7±5.5	6.8±2.1	6.7±2.1	5.8±1.7	5.6±1.3

Table VI

Dinamics of plasma progesterone level over 42 days post partum

Time of sampling	Plasma progesterone level (ng/ml) $\bar{x}\pm S$	
Group "A" (pregnant cows, n=6)		
Between weeks 0 to 2	0.76± 0.3	(n=67)
2 to 3	0.80± 0.34	(n=12)
4 to 5	2.18± 1.17	(n=12)
5 to 6	2.19± 1.31	(n=12)
Length of service period (days)	85.08±27.61	
Group "B" (non-pregnant cows, n=6)		
Between weeks 0 to 2	0.76±0.45	(n=23)
2 to 3	0.80±0.33	(n=6)
4 to 5	1.08±0.53	(n=6)
5 to 6	1.65±1.24	(n=6)
open days: 192—200		

preceded by fluctuation of the progesterone level between 1 and 3 ng/ml, signifying a gradual onset of ovarian function.

The effect of regular exercise during the third trimester of pregnancy are shown in Table VI.

Losses and certain reproduction-biological data are compared between two groups of cows walked (group II) and not walked (group I) a distance of 2800—3000 m on each day of the third trimester.

The daily exercise, along with dietary measures (increased daily hay ration, but reduced corn silage ration) exerted through the stimulation of ruminal digestion an advantageous influence on the protein and energy metabolism. Also, the muscle work involved in the exercise promoted the

Table VII

Health and reproduction-biological parameters of cows exercised and not exercised from the third trimester of pregnancy until calving

	Losses by death, emergency slaughter or culling (%)	Length of service period (days)	Fertility index
Group 1 (control) (n = 341) observed from Dec. 1, 1976 to May 31, 1977	21.4	141±86	3.3±1.8
Group II (exercised from Dec. 1 to May 31, 1978) n = 291	12.4	113±59 P<0.001	2.5±1.6 P<0.001

Percentual distribution of losses (percentage for group 1 in brackets)

	Hepatic, renal or digestive organ disease	Other pathological alterations	Infertility	Udder disease
Spontaneous death	4 (8)	6 (6)		
Emergency slaughter	5 (11)	8 (21)		
Culled			8 (8)	6 (19)
Total loss:	37 (73)		12.7% (21.4%)	

synthesis of glycogen from the lactic acid released through the Cori pathway and thus resulted in an increase of the hepatic glycogen reserves. Further, the exercise became expressed in a decreased frequency of difficult calvings, puerperal losses and emergency slaughters, as well as in a significant shortening of the service period and a decrease of the fertilization index. Total losses could thus be reduced by more than 40%, and the sick animals usually made an easy recovery.

Conclusions

In accordance with other investigators, we have found that in normal conditions SGOT level begins to rise a few days before calving and reaches its physiological peak, or a slightly higher value, during the first days post partum. Peripartal structural and functional alterations of the liver, consequent upon ruminal dysfunction and metabolic imbalance in advanced pregnancy, account for a considerable rise in the SGOT well before calving and for a simultaneous decrease in the serum cholesterol level. The diagnostic importance of changes in these two parameters 10 days before calving has been confirmed by our own observations. It should be noted that cows

5-year-old or older showed during calving and for about 10 subsequent days a significant by increased SGOT and a significantly decreased serum cholesterol level compared to younger animals of the same population. The older cows also had significantly lower inorganic P levels. These phenomena suggest a predisposing role of age and the pathogenetic importance of reduced hepatic detoxifying capacity in the development of the partus syndrome. The peripartal reduction of serum cholesterol showed a positive correlation with the length of the service period. Persistence of reduced cholesterol for 24—25 days post partum is of prognostic value in that it signifies prolongation of the service period and calving interval, a circumstance also indicated by the very low blood progesterone level of such animals (HARASZTI et al., 1979).

The sexual cycle, which ceases to function from the beginning of gestation, does not immediately reappear after calving. Normal ovarian function and, through it, the new cycle become restarted gradually, in the process of the so-called "sexual convalescence". Appearance of the first normal post-calving oestrus was preceded between 4—6 weeks after partus by a considerable fluctuation in the plasma progesterone level, the extreme values being: $2.18 \pm \pm 1.17$ and 2.91 ± 1.31 ng/ml. In this condition the length of the service period was 85.08 ± 27.61 days. Those cows, however, which had had plasma progesterone levels of 1.08 ± 0.53—1.65 ± 1.24 ng/ml between the post-calving weeks 4—6, had their first fertile oestrus at a later time, and the number of their open days increased to 192—200, owing to various types of ovarian and uterine disfunction.

Demonstration of shifts in the differential leucocyte count and of rise of glucocorticoid levels immediately before, and especially during calving have substantiated the relevance of partus stress. During calving, the total leucocyte count, and the neutrophil count rose to a maximum, while the eosinophil count fell to a minimum, and the serum glucocorticoid level rose abruptly. The disease-precipitating effect of stress can be regarded as a major pathogenetic factor in the development of the partus syndrome. Uncomplicated reproduction can be promoted and partus stress and related metabolic disorders can be reduced by regular exercise of the pregnant animals, particularly during the third trimester. Observations on a total of 632 cows have shown that daily exercise during the dry period accounted not only for a notable reduction of puerperal losses and emergency slaughters, but also for a significant abbreviation of the service period, reduction of the fertilization index and easy uncomplicated recovery of the sick animals.

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Summary

SGOT and differential leucocyte counts were followed up for 8 days both before and after calving in 45 Holstein-Friesian cows. In addition, in 17 of them cows serum glucocorticoid level, in 18 plasma progesterone level, was followed up for two and six weeks post partum, respectively. Progesterone was measured by radioimmunoassay. In a Hungarian Red Pied herd, 104 cows, serum cholesterol was followed up from the day of calving through 25 days. To check the adverse influence of confinement during the dry period, dry cows of a Holstein-Friesian herd were walked daily over a distance of 2800—3000 m, to compare the losses, the length of service period and the mean fertilization index with similar data of the previous year, when the animals were not exercised.

Attempts at identifying the causal factors of the partus syndrome have led to the following conclusions. Changes of the SGOT and serum cholesterol levels make possible a prognostic diagnosis of imminent partus syndrome as early as the time before calving. Cows 5-year-old or older showed a significant increase in SGOT during calving and on 10 subsequent days, along with a significant decrease in serum cholesterol, compared to younger animals of the same population. Low peripartal cholesterol levels showed a positive correlation with the length of service period. Onset of the first fertile oestrus after calving was preceded by fluctuations of plasma progesterone between 2.18 ± 1.17 and 2.91 ± 1.31 ng/ml in weeks 4 and 6 post partum. Those cows having only 1.08 ± 0.53 and 1.65 ± 1.24 ng/ml serum cholesterol in the same period had their first fertile oestrus later. Shifts of the differential 632 exercised cows showed, comparing to the non-exercised controls, decreased puerperal losses and emergency slaughters, shorter service period and lower fertilization index.

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STUDIES ON PORCINE ADRENOCORTICAL FUNCTION.

I. CIRCADIAN VARIATIONS IN, AND EFFECT OF ACTH TREATMENT ON, THE PERIPHERAL PLASMA CORTISOL LEVEL

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It is known from the investigations of SELYE (1973) that animals respond to the increase and/or prolongation of environmental stimuli by rise of the blood glucocorticoid level. Precise data on the quantitative changes in adaptive hormones have been obtained chiefly by the RIA and CPBA methods, but the phenomenon has been little studied in swine, and the few experiments reported in this species were based mainly on the less specific fluorometric technique.

This prompted us to study with the RIA technique the influence of given environmental stress factors on the adrenocortical function (ACF) of growing pigs, as assessed from the degree and duration of concentration increase of glucocorticoid in the peripheral blood.

First the physiological fluctuations of plasma cortisol were determined in growing pigs kept under optimal microclimatic conditions and fed *ad libitum*. To this end, the circadian variation of peripheral cortisol and its concentration increase induced at two levels of exogenous ACTH were measured.

Materials and methods

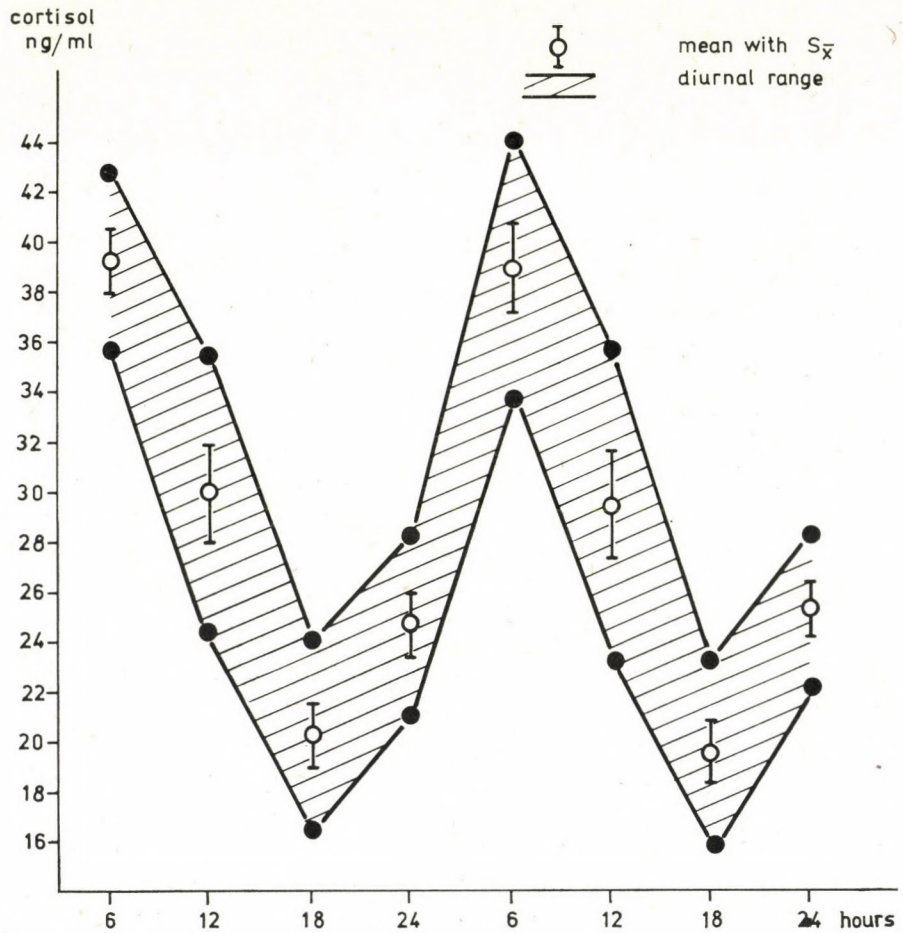
Hungarian Large White × Dutch Landrace F_1 pigs were used in the experiments. The animals were 30 days of age at the start and 180 days old at the end of the experimental period. During this time the average body weight of the pigs increased from 7.7 kg to 57.3 kg. The experiments were performed under controlled climatic conditions. The pigs were grouped by fours in flatdeck cages equipped with feeding trough and nipple drinker. A pig diet containing 88.6% dry matter, 22.4% crude protein, 18 430 kJ/kg brutto energy and 12 250 kJ/kg metabolizable energy (ME) was fed throughout the period of the experiment.

The cortisol concentration was determined with the Biolab Cortisol Biokit (Brussels), in plasma obtained from heparinized blood samples withdrawn from the cranial hollow vein.

a) Determination of the circadian variations of plasma cortisol in growing pigs

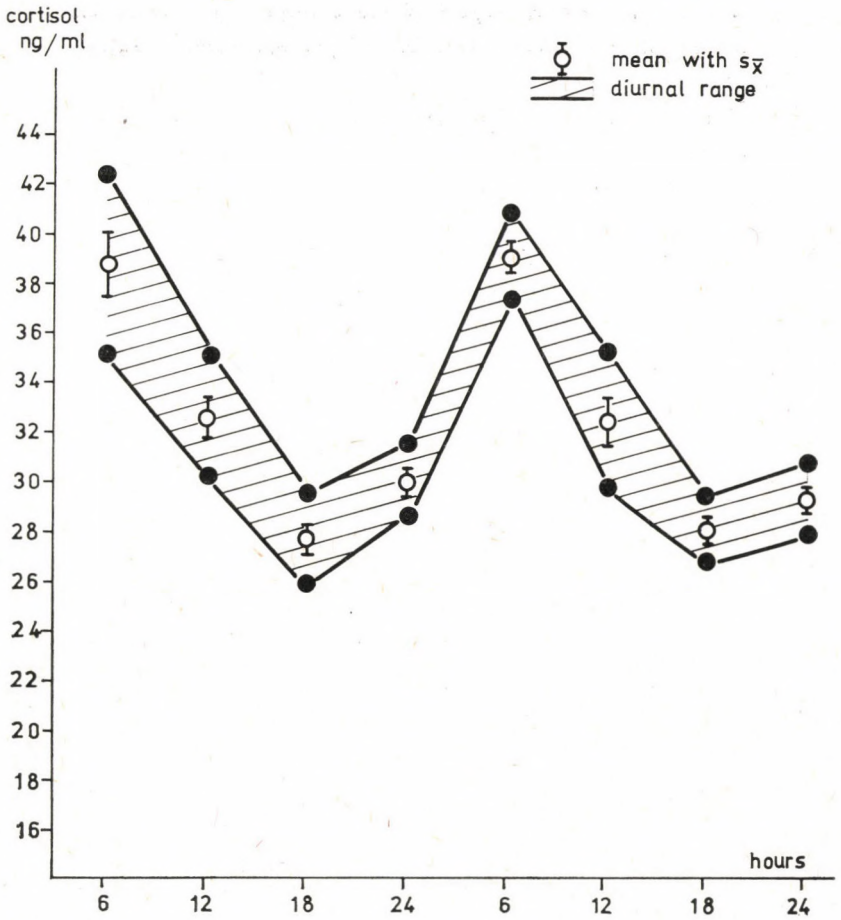
The plasma cortisol level of 8 growing pigs, kept in an optimum microclimatic environment and fed *ad libitum*, was determined in blood samples withdrawn at 6 a.m., noon, 6 p.m., and midnight on two consecutive days, on two occasions separated by an interval of 80 days. The main parameters of the experiment are shown in Table I, and the results are presented in Figs 1, 2, and 3.

Figure 1 shows the typical circadian fluctuation of the plasma cortisol level at the average age of 50 days. The highest concentration was measured at



$\bar{x}$	39.2	29.9	20.2	24.6	38.9	29.4	19.5	25.2
SD	3.6	5.6	3.8	3.6	5.2	6.3	3.7	3.1

Fig. 1



$\bar{x}$	38.8	32.7	27.8	30.1	39.2	32.6	28.2	29.5
SD	3.6	2.4	1.8	1.4	1.7	2.7	1.3	1.4

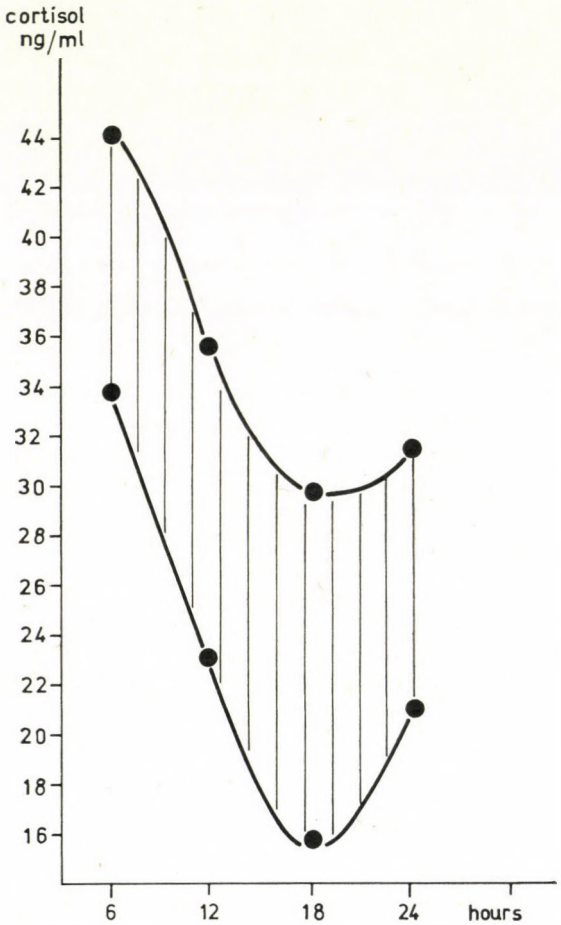
Fig. 2

6 a.m. the lowest one at 6 p.m.; the concentration difference was significant at the $P<0.001$ level. The average morning level was about twice as high as the average afternoon level. Analysis of the standard deviation has indicated that during the growing age the plasma cortisol level of pigs may vary between 16 and 44 ng/ml without any notable environmental stress.

Fig. 2 indicates essentially the same tendency of concentration change, but the difference between the morning and afternoon values was less pronounced (41%) than in the first sampling period. The difference between the highest morning levels and lowest afternoon cortisol levels was signif-

icant at the $P<0.001$ level. Analysis of the standard deviation showed that at 130 days of age the circadian variation of plasma cortisol ranged between 26 and 42 ng/ml.

The circadian fluctuations of the plasma cortisol level measured at 50 and 130 days of age were compared to assess the average range of variation for the total period studied. Comparison of the limit values of the standard deviations (Fig. 3) showed that the circadian variation of plasma cortisol ranged between 16 and 44 ng/ml in clinically healthy, 50 to 130 days old growing pigs kept under optimal microclimatic conditions and fed ad libitum.



Diurnal	44.1 –	35.5 –	29.6 –	31.4 –
range	– 33.7	– 23.1	– 15.8	– 21.0

Fig. 3

Table I

Main parameters of the experimental study of circadian variations in blood cortisol

Parameters	First experiment	Second experiment
Age, days	50	130
Average body weight, kg	15.2±0.7	51.3±1.7
Feeding	*ad libitum*	*ad libitum*
Ambient temperature, °C	26.0	19.3
Air humidity, rel. %	58	50
Illumination, lux	200	200

Table II

Main parameters of the experimental study of ACTH influence on peripheral plasma cortisol level

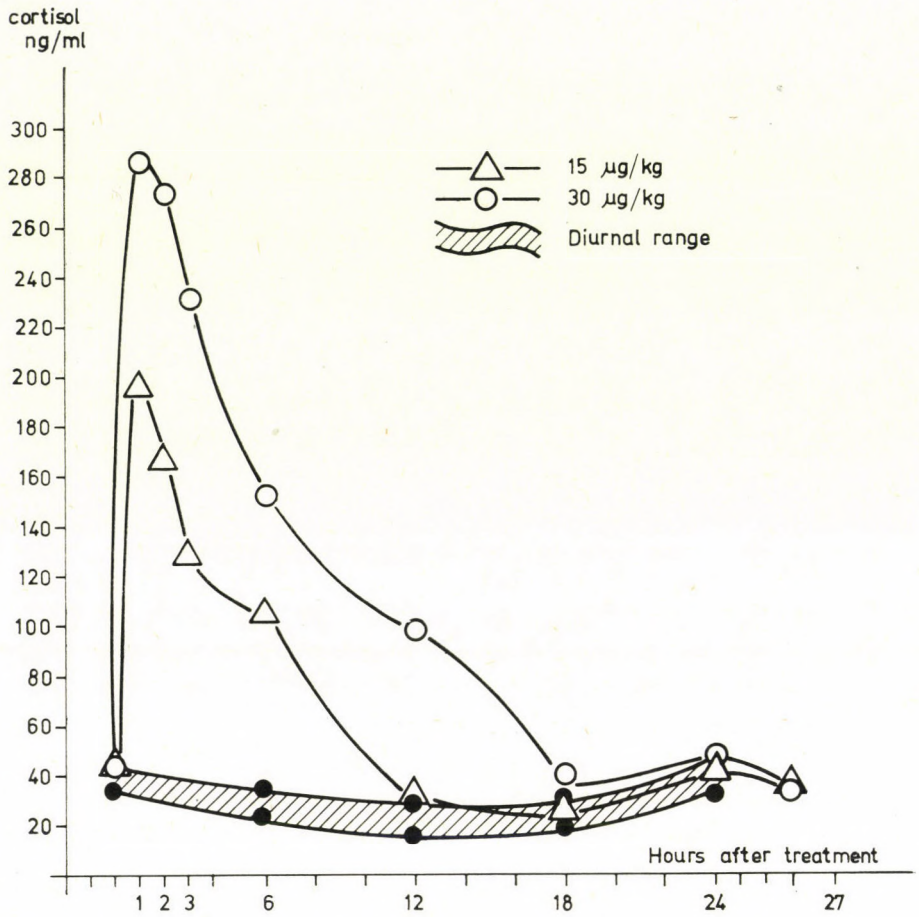
Age, days	65
Average body weight, kg	23.4±1.3
Feeding	*ad libitum*
Ambient temperature, °C	24.0
Air humidity, rel. %	60

b) Effect of exogenous ACTH on the adrenocortical function (ACF) of growing pigs

Growing pigs were examined for the degree and duration of cortisol elevation in response to a single treatment with an ACTH depot preparation.

The Cortrosyn Depot preparation of N.V. Organon (Oss, Holland) was used. One ml of this preparation contains 1 mg of a polypeptide the amino-acid sequence of which in the same as that of the first 24 amino acid residues of human ACTH. Two groups of five 65 days old pigs each, kept in an optimal microclimatic environment and fed *ad libitum*, were treated with a single i.m. dose of 15 and 30 μg/kg ACTH, respectively. The cortisol level was determined in plasma samples taken before ACTH-treatment, and 1, 2, 3, 6, 12, 18 and 24 hrs thereafter. The main parameters of the experiment are shown in Table II.

Figure 4 shows that the administration of ACTH was followed by a considerable rise in plasma cortisol at both dose levels. One hour after treatment, the pigs given 15 and 30 μg/kg ACTH showed a 455 and 641% increase in peripheral cortisol, respectively, over the upper limit of circadian variation. However, despite the use of a depot preparation, the cortisol concentration



		1	2	3	6	12	18	24	27
$\bar{x}$ 15 µg	43.0	195.9	166.8	129.2	106.5	31.0	27.0	42.3	39.1
SD	3.8	7.8	20.2	23.7	24.1	5.9	5.4	2.4	2.8
$\bar{x}$ 30 µg	44.6	284.0	273.3	230.6	152.0	99.3	38.7	47.4	34.3
SD	5.8	29.1	44.3	33.8	16.5	31.1	9.7	3.2	5.8

Fig. 4

tended to decrease steadily after the one-hour peak, until it returned to the initial value 12 and 18 hrs after administration of the lower and higher dose of ACTH, respectively.

Discussion

It was shown first by HEAP et al. (1966), later by HOLZBAUER and NEWPORT (1969), that the predominant glucocorticoid hormone of the pig is cortisol (hydrocortisone), against corticosterone in rodents (BUSH, 1953). Cortisol determination is, therefore, the primary approach to the functional study of the porcine adrenal cortex.

The present studies have revealed a considerable circadian variation of the cortisol concentration in the peripheral blood of pigs. This accords well with the observations of other authors (BOTTOMS et al., 1972; FAVRE and MOATTI, 1977; WHIPP et al., 1970) in pigs and other domestic animals (FULKERSON and TANG, 1979; LARSSON et al., 1979; RYNBERK et al., 1968; ZOLOVICK et al., 1966), as well as on humans (NICHOLS and TYLER, 1967). On cortisol determination with CPB technique in three blood samples daily from seven 3—6 months old SPF hybrid pigs, WHIPP et al. (1970) measured the lowest level (6 ng/ml) at midnight, and the highest level at 8 a.m. (24 ng/ml). In blood samples from Hampshire×Yorkshire F_1 pigs weighing on the average 90 kg, TOPEL et al. (1973) measured mean plasma cortisol concentrations of 41.2, 31.5 and 33.1 ng/ml at 8.0 a.m., 16.0 p.m. and midnight, respectively. FAVRE and MOATTI (1977) also used MURPHY's (1967) CPB technique to assess the circadian variation of the cortisol concentration in blood samples taken from the jugular vein of four 8 weeks old pigs, 12—15 kg in weight, at 2.0 a.m., 8.0 a.m., 2.0 p.m. and 8 p.m.; the levels found were 12.7, 20.2, 20.7 and 5.2 ng/ml, respectively. Like the cited authors, we, too, observed peak ACF in the morning, and a lower level of cortisol secretion in the afternoon. However, we found higher absolute values of plasma cortisol in our 50—130 days old pigs than FAVRE and MOATTI (1977) and WHIPP et al. (1970), presumably owing to the greater sensitivity of the RIA technique used by us. We observed a greater circadian variation of plasma cortisol in the younger than in the older age under the given conditions of experiment. The difference might be attributed simply to the progressing age and increased body weight, but the continuous illumination employed during the 80-day interval between the two sampling periods also might play a role in it. The continuous illumination did not, however, eliminate the circadian variation of ACF; it follows that factors unrelated to light are also involved in the control of the diurnal rhythm of the hypothalamus—pituitary-adrenocortical system.

As far as we are informed, the physiological and biochemical mechanisms underlying the circadian variation of cortisol and ACTH secretion have not yet been studied in pigs. SCAPAGNINI's (1978) rat experiments have indicated an inverse relationship between the hypothalamic noradrenaline (NA) synthesis and the secretion of ACTH, *i.e.* of glucocorticoids. It appears that inhibition of the NA synthesis causes a considerable rise in the peripheral

glucocorticoid level independently of the latter's actual circadian variation. At the same time a positive correlation has been demonstrated between the serotonine (5-hydroxytryptamine) content of the limbic systems and the blood glucocorticoid level. Notwithstandingly, MÜLLER et al. (cit. SCAPAGNINI et al, 1978) observed a marked circadian variation of the cerebral serotonine content in certain domains of the brain.

Treatment with exogenous ACTH gave rise to a rapid concentration increase in plasma cortisol. In the given conditions of experiment, the highest cortisol level was measured during the first hour after ACTH administration, but this did not, presumably, correspond with the peak level characteristic of the dose. BLATCHFORD et al. (1978) reported that 6 to 10 weeks old Large White gilts treated with 1 IU/kg ACTH showed a twofold increase in the peripheral glucocorticoid level within 2 min after hormone administration, and a peak level within 8 min. A similarly quick response was observed by FAVRE and MOATTI (1977). We, too, found a direct relationship between the applied dose of ACTH and the consequent rise in the peripheral glucocorticoid level but, surprisingly, the peak was soon followed by a rapid drop despite the use of a depot preparation.

It follows from our experiments that the ACF of growing pigs may vary considerably even under optimal keeping and feeding conditions, *i.e.* without any environmental stress. As the daily maximum and minimum concentrations of blood cortisol differed significantly, it is imperative to take into consideration the circadian variation of blood cortisol, *i.e.* the time of blood sampling, in plasma cortisol determinations performed for stress diagnosis. The ACTH-induced rise of plasma cortisol serves as a useful reference value in judging the impact of environmental stress factors on the growth and productive performance of pigs.

Summary

The peripheral cortisol levels of 8 Hungarian Large White × Dutch Landrace F_1 pigs were determined by the RIA technique at 6.00 a.m., noon, 6.00 p.m. and midnight on two consecutive days each at two different ages of life, *viz.* 50 and 130 days, to assess the circadian variation of adrenocortical function in optimal keeping and feeding conditions, *i.e.* without any environmental stress. In another experiment two groups of 65-day-old pigs, five animals in each group, were treated i.m. with 15 and 30 μg/kg ACTH, respectively, to determine the influence of exogenous ACTH on blood cortisol in optimal environmental conditions.

The peripheral plasma cortisol level showed a marked circadian variation which tended to decrease with progressing age. The adrenocortical activity was highest at 6.00 a.m. and lowest at 6.00 p.m. The average range of the circadian variation of plasma cortisol was assessed as 16—44 ng/ml in the age group studied.

Treatment with 15 and 30 μg/kg ACTH accounted for a 455 and 641% rise of peripheral plasma cortisol within one hour, followed by return to the baseline after 12 and 18 hrs, respectively.

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STUDIES ON PORCINE ADRENOCORTICAL FUNCTION

II. EFFECTS OF THE AMBIENT TEMPERATURE AND RESTRICTED FEEDING ON THE PERIPHERAL CORTISOL LEVEL

By

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The glucocorticoids act primarily on the carbohydrate metabolism. They enhance glucose formation and hepatic glycogen synthesis, act towards elevation of the blood glucose level, accelerate the degradation of proteins, and inhibit amino acid uptake and protein synthesis in all organs except the liver. Growing pigs are able to adapt themselves to extreme environmental stimuli, but only through the utilization of surplus energy (KOVÁCS, 1971; RAFAI, 1976). Since an increased glucocorticoid production presupposes the mobilization of the organism's energy reserves, the hypothalamic-hypophyseal-adrenocortical system plays an important role in adaptation to a non-optimal environment.

The present experiments were performed to determine the degree of modification of the adrenocortical function by nutritional and microclimatic factors responsible for mobilization of energy reserves.

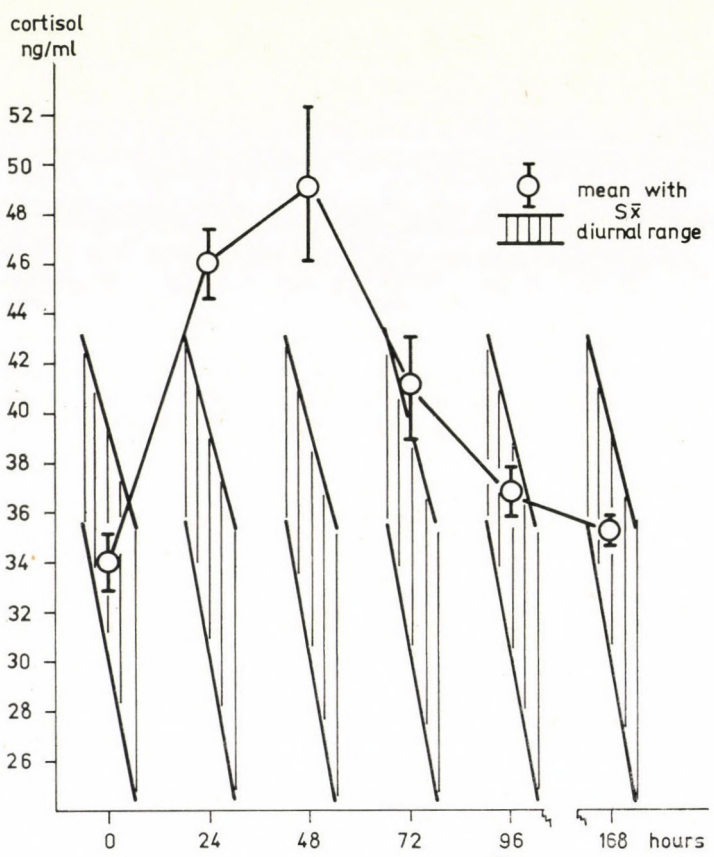
Materials and methods

Four experiments were performed in the climatic chambers of this Institute. The experimental animals, the conditions of their keeping and feeding, and the methods of blood sampling and cortisol determination were the same as described in the first publication of this series (RAFAI and FODOR, 1980). Each experiment was preceded by a 7—14 day period during which the optimum microclimatic environment and *ad libitum* feeding were furnished under consideration of the age and body weight of the animals. Weight gain and feed consumption were determined at weekly intervals during this preliminary period. Each experiment lasted 7 days, and was started on a Monday. After blood sampling and weighing at 9 a.m., the plane of nutrition and the microclimate were altered according to the purpose of the experiment. Blood samples were collected for cortisol determination 24, 48, 72, 96 and 168 hrs after the experiment had been began, always at 9.00 a.m. After conclusion of the experiment, weight gain and feed consumption were determined again, and the animals were returned to optimum microclimatic conditions and *ad libitum* feeding. The main parameters of the experiments are shown in the enclosed Tables.

Results

a) Effect of restricted feeding on the adrenocortical function of pigs kept in the optimum microclimatic environment.

Eight growing pigs, on average 105 days old and 47 kg in weight, were examined for the effect of restricted feeding on adrenocortical function. The main parameters and results of the experiment are shown in Table I and Fig. 1. Table I shows that on the *ad libitum* feeding regime employed during the preliminary period, the mean daily metabolizable energy (ME) uptake was 1420 kJ/kg$^{0.75}$; the average daily weight gain and feed conversion rate



$\bar{x}$	34.0	46.0	49.2	41.1	36.8	35.2
SD	3.1	46.1	8.8	5.6	2.9	1.5
P to basal		<0.001	<0.001	<0.01	NS	NS

Fig. 1

Table I

Main parameters of the experiment on the effect of a low plane of nutrition on plasma cortisol in the optimum microclimatic environment

Parameters	Preliminary period	Experimental period
Age days	105	112
Mean body weight, kg	47.1±1.8	45.4±1.7
Feeding	*ad libitum*	maintenance ration
Mean ME uptake, kJ/kg$^{0.75}$/day	1420	440
Feed conversion rate, kg/kg	2.5	0
Average daily weight gain	808	−245
Ambient temperature, °C	20	20
Air humidity, rel. %	60	60

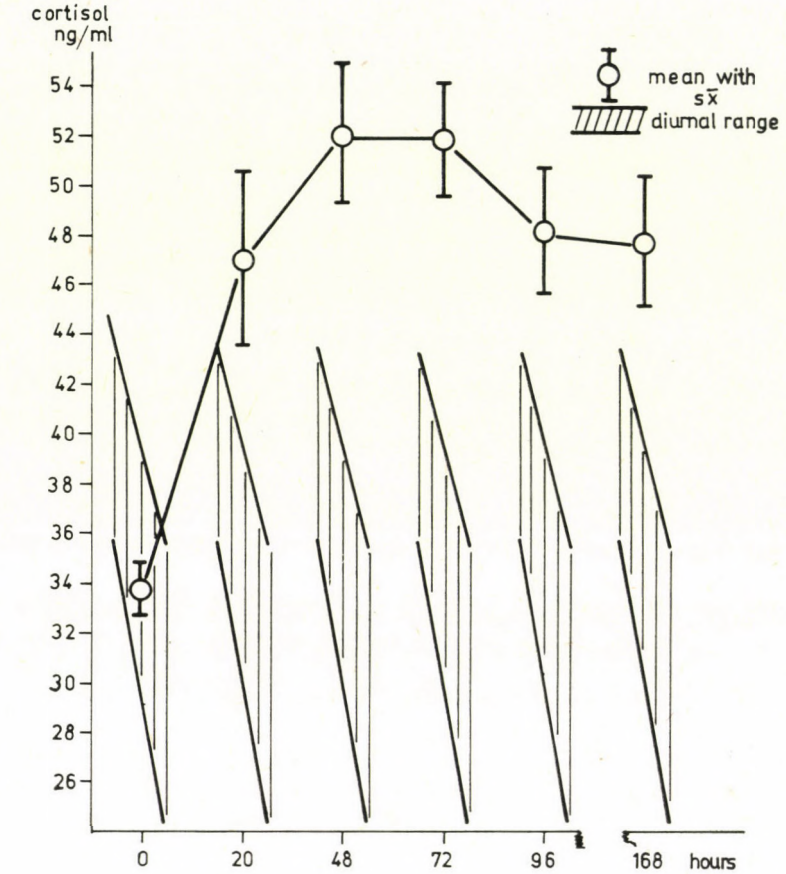
Table II

Main parameters of the experiment on effect of cold environment on the peripheral cortisol level of ad libitum fed pigs

Parameters	Preliminary period	Experimental period
Age, days	67	74
Mean body weight, kg	33.9±1.7	35.3±1.7
Feeding	*ad libitum*	*ad libitum*
Mean ME uptake, kJ/kg$^{0.75}$/day	1480	1550
Feed conversion rate, kg/kg	2.65	8.97
Average daily weight gain	611	201
Ambient temperature, °C	22	5
Air humidity, rel. %	60	80

were correspondingly favourable. Restricted feeding accounted for an average daily weight loss of 245 g.

Figure 1 shows the plasma cortisol levels at the indicated times. The mean cortisol concentration measured before feeding was reduced had been intermediate between the two circadian extremes (RAFAI and FODOR, 1980). Under the restricted feeding regime the plasma cortisol level rose significantly over the baseline within 24 hrs, and increased further until 48 hrs, where after it tended to decrease despite the unaltered conditions. Although the increase in plasma cortisol was statistically significant ($P<0.001$—$P<0.01$) during the first three days, the maximum, measured at 48 hrs, exceeded the upper limit of the circadian variation only by about 12%.



$\bar{x}$	33.7	47.0	52.0	51.8	48.1	47.7
SD	3.0	10.4	8.5	6.9	7.5	7.7
P to basal		< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

Fig. 2

b) Effect of low ambient temperature on the adrenocortical function of *ad libitum* fed pigs.

The effect of reduction of the ambient temperature to +5 °C on the adrenocortical function was studied in 9 pigs. The main parameters and results of the experiment are shown in Table II and Fig. 2.

Table II shows that the ME uptake did not notably increase over the baseline in the cold environment. This accounted for a significant ($P<0.001$) decrease in weight gain, and a more than threefold increase of the feed conversion rate. Simultaneously, the plasma cortisol level increased ($P<0.001$) over the baseline within the first 24 hrs of cold exposure, reached maximum

Table III

Main parameters of the experiment on effect of cold environment plus restricted feeding on the peripheral cortisol level of growing pigs

Parameters	Preliminary period	Experimental period
Age, days	80	87
Mean body weight, kg	40.5±1.6	37.2±1.2
Feeding	*ad libitum*	maintenance ration
Mean ME uptake, kJ/kg$^{0.75}$/day	1345	440
Feed conversion rate, kg/kg	2.70	0
Average daily weight gain	685	−478
Ambient temperature, °C	20	5
Air humidity, rel. %	60	80

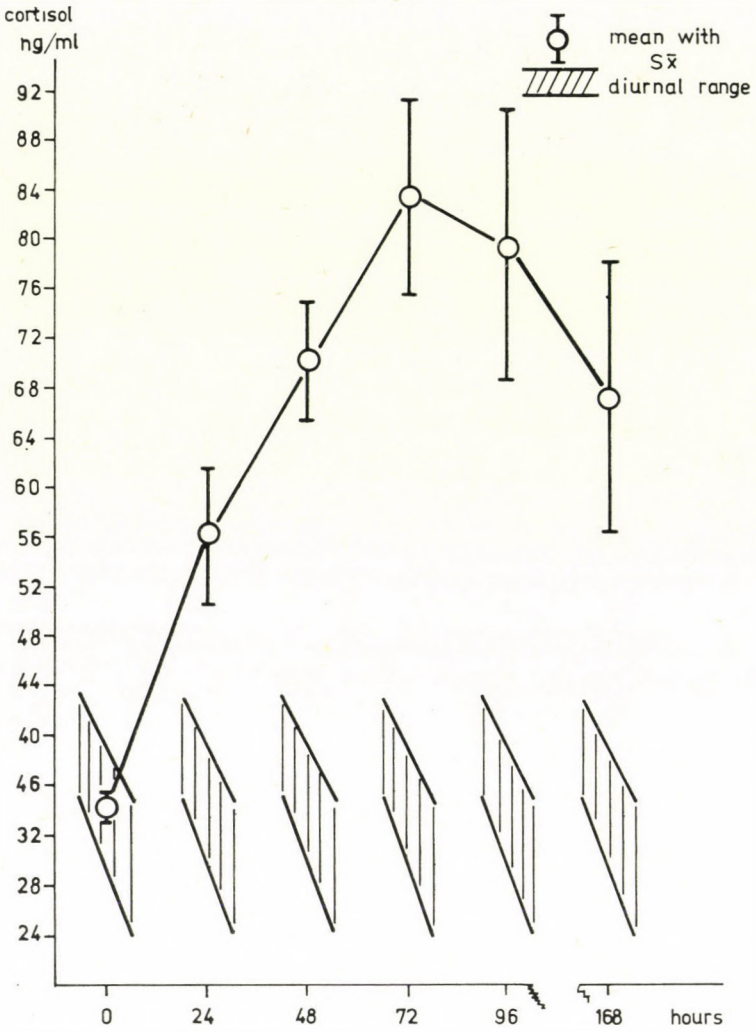
between 48 and 72 hrs, and subsequently tended to decrease. In this experiment, too, the absolute increase in plasma cortisol was only about 18% relative to the upper limit of circadian variation.

c) Effect of low ambient temperature and restricted feeding on the adrenocortical function of growing pigs.

The effects of low ambient temperature (+5 °C) plus reduced feed supply (440 kJ ME/kg$^{0.75}$ daily) on the adrenocortical function were also studied in 9 pigs. The main experimental parameters and results are shown in Table III and Fig. 3.

Simultaneous cold exposure and reduced feeding accounted for an average daily weight loss of 478 g. The peripheral plasma cortisol level increased ($P<0.001$) over the baseline within 24 hrs, and continued to show a highly significant elevation throughout the 7-day period of experiment. The adrenocortical activity was the highest on the third day of cold exposure, then it tended to decrease. The cortisol level measured at 72 hrs was considerably lower than the concentration increase inducible with exogenous ACTH, yet it exceeded the upper limit of circadian variation by 90%.

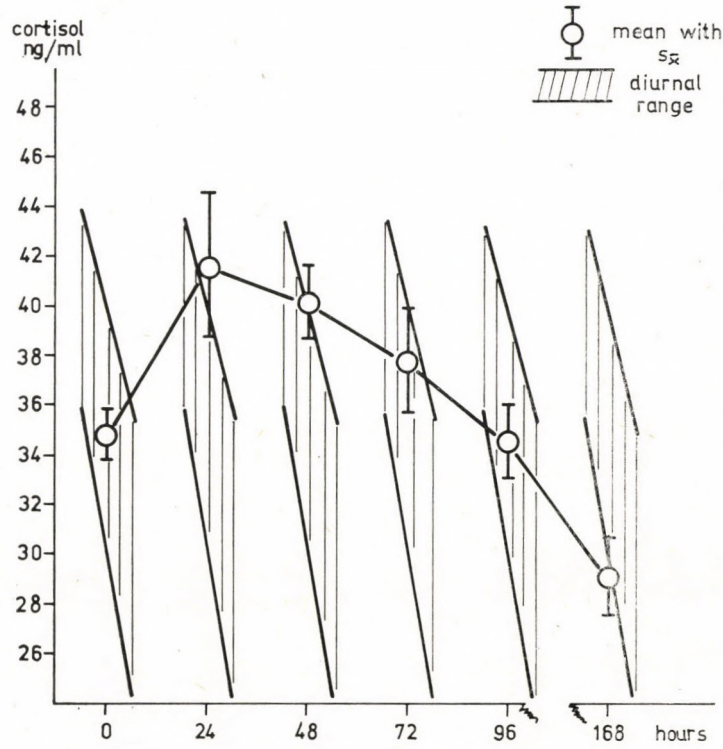
After conclusion of the 7-day experiment, the ambient temperature was raised to 20 °C, relative humidity to 60%, and the feed was again supplied *ad libitum*. The post-exposure changes in plasma cortisol were followed up in blood samples taken 24, 48, 120, 144 and 168 hrs after optimization of the microclimatic and feeding conditions. At 24 hrs, all pigs except one showed a reduced plasma cortisol, *viz.* 39.6±1.2 ng/ml on the average. This concentration was still significantly ($P<0.001$) higher than the mean level (34.2±1.0 ng/ml) measured at the end of the preliminary period, but it was already significantly lower ($P<0.05$) than the mean value (67.3±10.6 ng/ml) found at 168 hours. By 48 hrs the mean cortisol level of 8 pigs had practically returned to



$\bar{x}$	34.25	56.14	69.34	83.44	79.24	67.35
SD	2.8	16.2	15.5	22.9	32.1	29.9
P to basal		< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

Fig. 3

the baseline, and persisted close to it. The single pig showing persistence of elevated cortisol despite optimization of the keeping conditions died in the 120th h. A plasma taken from the animal in the premortal stage by cardiac puncture contained 141.1 ng/ml cortisol. At postmortem examination we found an extensive catarrhal pneumonia involving the right cardial and apical lobes of the lung.



$\bar{x}$	34.8	41.6	40.1	37.8	34.6	29.1
SD	2.8	8.4	4.2	5.6	4.4	4.2
P to basal		0.05	0.01	NS	NS	0.01

Fig. 4

d) Effect of supra-optimal ambient temperature on the adrenocortical function of growing pigs.

We raised the ambient temperature to 33 °C to study the effect of warm environment on 8 fattening pigs. The pigs weighed on the average 54.6 kg. Blood samples were taken on days 1, 2, 3 and 7 of the experiment, always at 9.00 a.m. The rectal and skin temperatures, and the respiration rate of the animals were measured at noon on each sampling day. The main experimental parameters are shown in Table IV, the results in Figs 4 and 5, and Table IV.

Keeping in the warm environment accounted for a 30.5% decrease of ME uptake compared to the preliminary period while the rectal and skin temperatures, and the respiration rate rose significantly ($P<0.001$) over the baseline despite reduced feed consumption. The plasma cortisol level tended to rise during the first 24 hrs of heat exposure, then it fell gradually. The increase in plasma cortisol over the baseline was significant ($P<0.05$ and

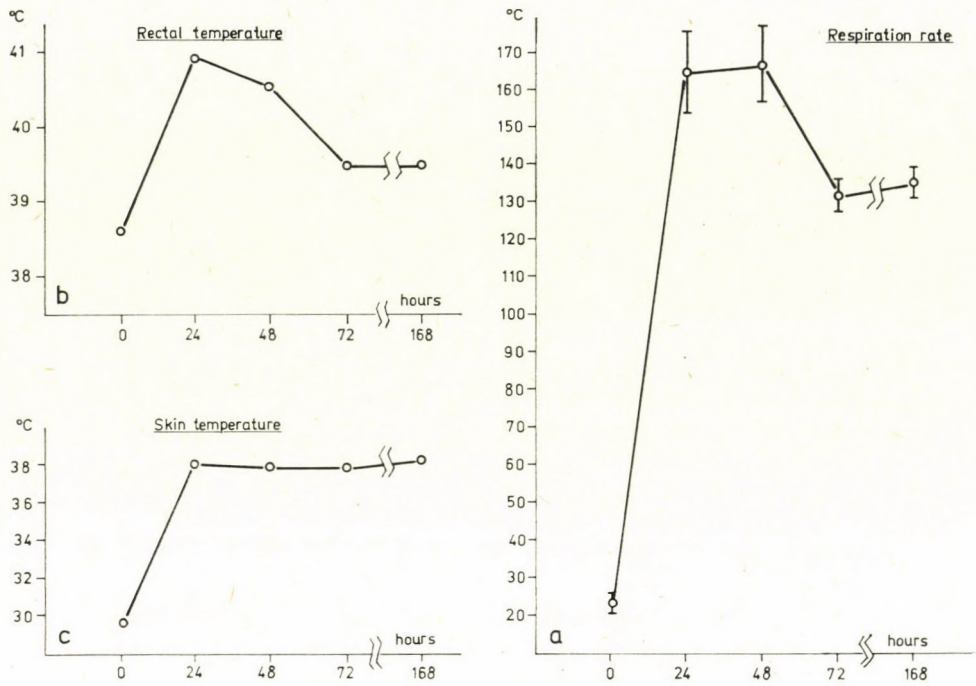


Fig. 5

Table IV

Main parameters of the experiment on the effect of warm environment on the peripheral cortisol level of growing pigs

Parameters	Preliminary period	Experimental period
Age, days	126	133
Mean body weight, kg	54.6±2.5	57.3±2.5
Feeding	*ad libitum*	*ad libitum*
Mean ME uptake, kJ/kg$^{0.75}$/day	1395	970
Feed conversion rate, kg/kg	2.92	4.31
Average daily weight gain	837	375
Ambient temperature, °C	20	33
Air humidity, rel. %	65	65

$P<0.01$) during the first 48 hrs, but the mean concentration remained within the limits of circadian variation. By day 7 of the heat exposure the cortisol level dropped ($P<0.01$) below the baseline recorded at the end of the preliminary period.

Discussion

The daily ME uptake by the growing pigs ranged between 1345 and 1480 kJ/kg$^{0.75}$ during the preliminary periods in which the optimum microclimatic environment and *ad libitum* feeding was furnished. CLOSE and MOUNT (1978) assessed the daily maintenance energy requirement of individually-caged growing Large White pigs, 20—50 kg in weight, as 440 kJ ME/kg$^{0.75}$ at a thermoneutral ambient temperature. The maintenance energy requirement is defined as the quantity of ME sufficient to maintain undisturbed life functions at zero energy balance. In view of this, it was surprising that the pigs reduced to a daily maintenance ration of 440 kJ ME/kg$^{0.75}$ showed a mean daily weight loss of 245 g during the 7-day period of the experiment. One reason may have been the group keeping, *i.e.* cageing by fours, which may have accounted in itself for an increase in the non-productive energy requirement. Consideration should also be given to the fact that the heat loss and the critical ambient temperature of the growing pig shows close correlation with the plane of nutrition (CLOSE and MOUNT, 1978), *i.e.* the lower the ME uptake the higher ambient temperature is required. At last but not at least, the Hungarian Large White × Dutch Landrace F_1 crosses used by us may have had, for genetic reasons, a greater maintenance energy requirement than purebred Large White pigs. It follows that the maintenance ration of 440 kJ ME/kg$^{0.75}$, supplied at 20 °C ambient temperature, did not meet the maintenance energy requirement of the pigs in the given conditions. Consequently, the animals obtained part of the necessary maintenance energy from their own energy reserves, by a mechanism in which the catabolic adrenocortical hormone cortisol obviously played an important role. It is known that cortisol promotes the breakdown of myoproteins, and the amino acids released during degradation are utilized by the visceral organs (liver, pancreas, intestines), intensely involved in protein metabolism, so that in the condition of undernutrition the integrated function of the latter presupposes loss of muscle protein (JAYA RAO, 1978). This can satisfactorily explain the slight rise in plasma cortisol shown by pigs kept at a low plane of nutrition at 20 °C ambient temperature.

According to our unpublished observations, the heat production of caged growing pigs, 30—35 kg in weight, was the lowest at 20—22 °C in the condition of feeding *ad libitum*. It follows that the 5 °C ambient temperature used in experiments *b)* and *c)* was about 15—17 °C less than the environmental temperature optimum. This accounts for a considerably increased heat loss, which is partly compensated by the animal through a decrease in the cutaneous and rectal temperature, and by behavioural thermoregulation. Nevertheless, the primary mechanism promoting maintenance of the homoeostasis in a cold environment is not so much the decrease of heat loss as the increased

heat production. The energy requirement of the greater heat production is met via an increased feed consumption. The pigs fed *ad libitum* in the cold environment showed only a minor increase in feed uptake during the 7-day period of experiment, but the average daily consumption of 1.81 kg feed corresponded to more than 5% of the average body weight. Since, however, the surplus consumption did not meet the energy requirements of increased heat production and growth, the consequent decrease in weight gain accounted for a notable decline in the feed conversion rate.

Ad libitum fed and undernourished pigs equally showed a rise of the peripheral plasma cortisol level during the first 72 hrs of cold exposure. From the fourth day on, the cortisol level tended to decrease, but continued to persist significantly above the baseline determined in the optimum microclimatic environment. Against this, MARPLE et al. (1972a) found no change in the plasma corticoid level with the modified CPB technique during cold exposure (4.4 °C — 46 r.h.%) of growing Chester White pigs fed *ad libitum*. In another experiment on growing Hampshire×Yorkshire F_1 pigs, also fed *ad libitum*, MARPLE et al. (1972b) even observed a significant decrease in total plasma corticoid (45±4 ng/ml), despite ACTH elevation, during a 7-day cold exposure (4.4 °C — 100 r.h.%), relative to controls (54±3 ng/ml) maintained in an optimal microclimatic environment (21 °C — 61 r.h.%). From this, MARPLE et al. concluded that the adrenocortical synthesis and/or release of corticoids was lower than required under the given conditions of experiment. Unlike MARPLE et al. (1972b), TOPEL et al. (1971) and BLATCHFORD et al. (1978) arrived at conclusions similar to ours. The last-cited authors observed a tenfold increase in ACTH and threefold increase in total plasma corticoid over normal during a 2-h exposure of Large White pigs weighing 15—20 kg to —5— —7 °C ambient temperature. Our own findings suggest that, among other neuro-endocrine changes, an increased glucocorticoid secretion plays a role in the cold adaptation of growing pigs either fed *ad libitum* or undernourished. Since, however, the partially starved animals showed a greater increase in peripherial plasma cortisol, there is reason to postulate that during the initial stage of adaptation to cold the energy-mobilizing specific effect of corticoid secretion predominates over its nonspecific increase under stress. We do not attribute the slight decrease in plasma cortisol, observed after 3 days of cold exposure, to exhaustion of the adrenal cortex, for in an earlier experiment (RAFAI and FODOR, unpublished) we were able to maintain an evenly high cortisol level by 10-day treatment with 1 mg ACTH per animal per day. We believe that the slight decrease in cortisol following upon its initial elevation signified adaptation to cold, since it was shown earlier (KOVÁCS, 1971; KOVÁCS et al., 1974) that the heat production of weanling pigs was highest during the initial days of cold exposure, then tended to gradually decrease.

Despite the decreased feed consumption and the increased respiration rate, the fattening pigs showed a significant rise in the rectal and cutaneous temperature in a warm environment. During the first 48 hrs of heat exposure, hyperthermia was associated with a significant, though minor, rise in the peripheral plasma cortisol level. This accords well with the experimental observations of ABERLE et al. (1974). In our experiment, the drop of plasma cortisol level ran parallel with the decreasing rectal temperature and respiration rate until, on the 7th day of the experiment, it fell significantly below the baseline measured at the conclusion of the preliminary period. This correlates well the cited results of MARPLE et al. (1972a).

The stress condition is the non-specific response of the organism to environmental stressors (SELYE, 1973). In this light, the stressor role of restricted feeding and of non-optimal microclimatic conditions employed in our experiments is self-explanatory. Nevertheless, only the combination of low feed supply with low environmental temperature induced a notable and durable rise in plasma cortisol. Further studies are required to clarify in which conditions of environmental stress can the rise of plasma cortisol be utilized as a stress marker.

Summary

The effect of non-optimal microclimatic conditions and/or restricted feeding on the adrenocortical function of growing pigs was studied in four experiments on Hungarian Large White × Dutch Landrace F_1 crosses at the age of 67 to 133 days. Reduction of the dietary energy supply to 440 kJ ME/kg$^{0.75}$ and of the ambient temperature to 5 °C for seven days represented a severe environmental stress inducing a considerable rise in the cortisol concentration in the peripheral blood plasma. Low plane of nutrition in the optimum microclimatic environment also gave rise to a significant increase in plasma cortisol during the first days, but by 96 h the blood level of the hormone had returned to a value within the range of physiological circadian variations. The adrenocortical function of the pigs also was significantly increased at ambient temperatures 15—17 °C colder or 10—13 °C warmer than the optimum, but the elevation of plasma cortisol was temporary and did not notably exceed the upper limit of circadian variation.

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RELATIONSHIP BETWEEN THE ASH CONTENT AND MICROSCOPIC HARDNESS OF SWINE BONES

By

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The so-called "leg weakness" or "bone weakness" syndrome has shown a rising incidence in large pig production units of several countries.

This prompted investigations into the causes of the performance-limiting movement disturbances (Behrens, 1961; Claus, 1962; Gängel and Stumpf, 1969; Grünhagen, 1969). The aetiological studies have been increasingly extended to the structural properties and chemical composition of bones (Dämmrich and Unshelm, 1972, 1975; Kerk, 1974; Meyer and Gröning, 1965; Nielsen, 1973; Stockland and Blaylock, 1973; Vaughan, 1971; Wentworth et al., 1971) and a suitable experimental approach has been found to micromechanical examinations.

The hardness of a solid material is defined as its resistance to the penetration of another — more solid — body. Hardness tests are thus based on the impression of a very hard body of well-defined shape (diamond cone, steel ball, etc.) into the body to be tested, by static or dynamic force, for a given time. Hardness is expressed in terms of the correlation between the force or work required for impression, and some parameter of the imprint produced. Imprints of microscopic size are conclusive of the microscopic hardness of the test material. This approach makes possible hardness measurements on very small blocks of bone.

The correlation between the microscopic hardness of bone tissue and the degree of osseous mineralization was first recognized by Carlström (1954), who demonstrated low, medium and high degree of mineralization in different parts of the human femoral bone and noted that microhardness tended to increase as the mineral content rose. His measurements were made on different points of a single Haversian system. Later Weaver (1966) and Jankovich (1972), demonstrated a correlation between ash content and microhardness of human and rat bones.

Materials and methods

We performed comparative studies on extremity bones of domestic pigs and a wild boar. The humerus (H), radius (R), ulna (U), metacarpus (Mc/IV), femur (F), tibia (T) and metatarsus (Mt/IV) of five 24-day-old piglets, eight 6-month-old fattening pigs, two 2-year-old sows, one 2.5-year-old boar and a 1.5-year-old wild boar, were used for microhardness testing.

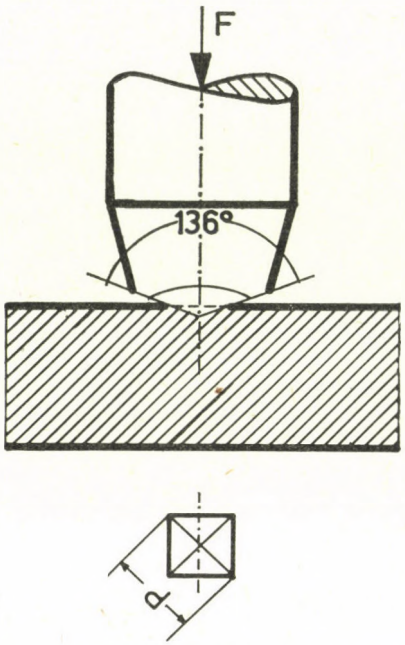

Fig. 1. Impression and imprint of the Vickers microhardness tester

After removal of the attaching muscles, the bones were fixed in 10% neutral formalin. All the specimens used for the determination of ash content, microhardness and structure were taken from the central part (diaphysis) of the bone.

For ash determination an 0.5—1.0 g specimen of the bone sample was defatted and incinerated. The ash content was expressed as per cent relative to the weight of the defatted bone.

For hardness testing of bones the procedure commonly used in the metal industry (penetration force, time of impression) had to be modified. The modified parameters of measurement were established on the basis of theoretical considerations, and practical observations made in preliminary studies. About 5-mm-thick bone specimens were cut in the horizontal plane, at a right angle to the bone axis. A square-based pyramidal diamond indenter, with an apical angle of 136°, was applied to the cut surface of the bone under a load of 0.3 kp (F). The diagonals of the imprint were measured to calculate its area (Fig. 1). The Vickers hardness (HV) of the bone was determined by measuring the hardness with a Zwick type 3202 microhardness tester. The HV number was calculated by dividing the load in kp with the impression surface in mm^2, using the formula

$$HV = \frac{F}{d^2} \text{ kp/mm}^2$$

where HV is the hardness measured, F the load, and d the diagonal of the imprint.

For structural studies, specimens a few mm in thickness were cut from the diaphysis at right angle to the bone axis, and the cut surfaces were polished first with coarse, then with more delicate, grades of grit paper. The polished surfaces were photographed.

Results

The values of ash content and microhardness determined in the cranial and caudal parts of the same femoral bone specimens are shown in Table I.

Table I

Sectorial variation of bone ash content and microhardness in the same specimen of porcine femoral bone

	Microhardness				Ash content (%)			
	Cranial side		Caudal side		Cranial side		Caudal side	
	n	x±s	n	x±s	n	x±s	n	x±s
Domestic pig 0.5-year-old	18	53.5±3.2	15	47.2±2.9	12	64.4±0.4	13	63.1±0.5
Sow, 2-year-old	7	76.6±4.2	9	61.6±7.2	8	66.8±1.2	9	64.3±1.0
Wild boar, 1.5-year-old	13	76.1±5.3	8	66.9±5.6	5	66.2±0.9	7	64.2±0.9

n, number of tests; x±s, mean±standard deviation.

The ash and microhardness measured in the central part from proximal to distal direction are presented graphically in Fig. 2. Age distribution curves for the same values are shown in Fig. 3.

Figure 4 shows the values of ash content and microhardness in different extremity bones of the same animal. The values shown in Figs 2—4 all apply to the cranial side of the bone.

Discussion

Table I and Figs 2 and 3 unequivocally indicate that the ash content and microhardness of the femur varied between the different sectors of the bone in 0.5—2-year-old domestic pigs and in the 1.5-year-old wild boar as well. The ash content and microhardness were always greater in the cranial than

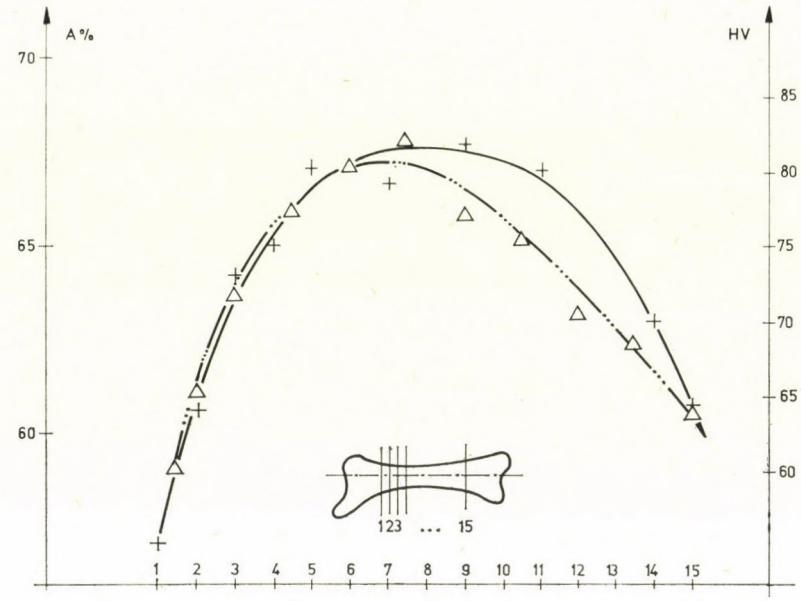

Fig. 2. Alterations of ash per cent (A%) and microhardness (HV) along the longitudinal axis of the femoral diaphysis (2.5-year-old pig). + HV; △A%

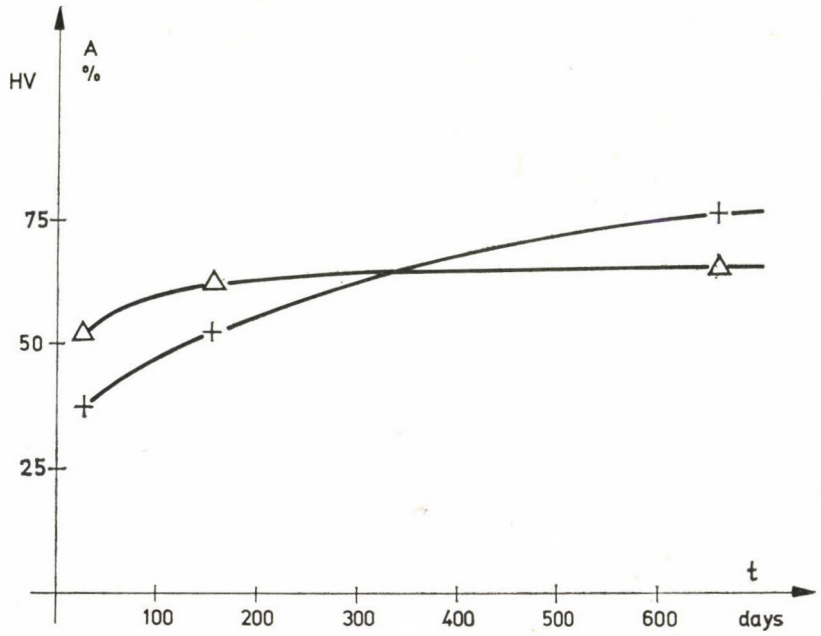

Fig. 3. Femoral ash per cent (A%) and microhardness (HV) as a function of age. + HV; △A%

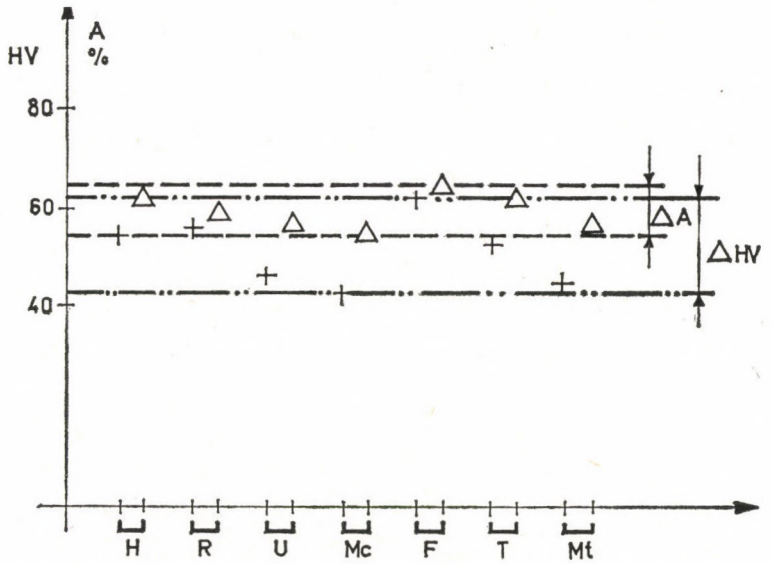

Fig. 4. Ash per cent (A%) and microhardness (HV) of different extremity bones of a half-year-old fattening pig. H, humerus; R, radius; U, ulna; Mc, metacarpus; F, femur; T, tibia; Mt, metatarsus; + HV; △A%

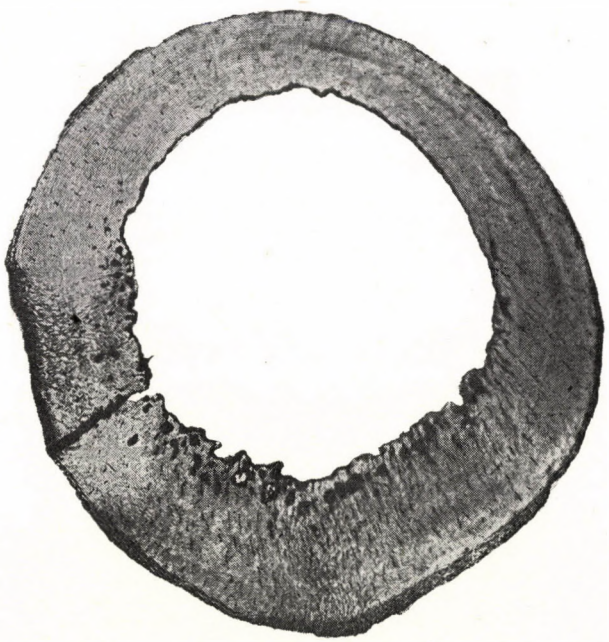

Fig. 5. Transversal section of the femoral diaphysis. ×3

in the caudal part, and both were highest in the middle height of the diaphysis. A positive correlation of variable degree was demonstrable between the ash content and microhardness of also other extremity bones (Fig. 4).

The present findings, along with observations of others on human bones (AMPRINO, 1958; EVANS and LEBOW, 1951; EWANS and BANG, 1967; WEAVER, 1966; YOON and KATZ, 1976) support the conclusion that mechanical strain and chemical composition of the bone play an important role in the latter's mechanical functions. Obviously, the inorganic components of the bone account primarily for resistance to compressive strength, while the occeous

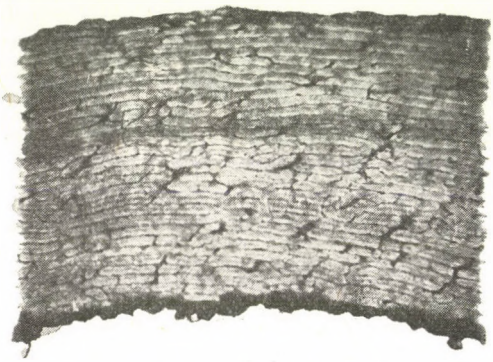

Fig. 6. Transversal section of the femoral diaphysis — cranial part. ×15

Fig. 7. Transversal section of the femoral diaphysis — caudal part. ×15

collagen accounts mainly for resistance to tensile strength. Bone hardness depends primarily on the amount of calcium salts, but the orientation of collagen fibres and the moisture content of the bone also play a role in it (AMPRINO, 1958). The quantity and chemical composition of the calcium salts become altered with biological aging (LENGYEL, 1976). In the initial stage of bone mineralization, an amorphous octa-calcium-phosphate is deposited in the osteoid tissue, but later crystalline compounds (hydroxyapatite) become predominant (POSNER, 1969). The osteons containing the bone minerals in a predominantly amorphous form are involved in bone metabolism, while those consisting predominantly of crystalline hydroxyapatite have a statical supporting function.

The adaptation of the bone to its mechanical function is shown in Fig. 5. The femur of the standing pig forms an angle of 60° with the horizontal plane. It follows that the mechanical strain of the bone differs between its cranial and caudal part. Figure 6 shows that the cranial part of the femoral diaphysis of the 0.5-year-old pig is formed by compact laminar bone tissue, with few if any, secondary osteons, while the caudal part is spongiform (Fig. 7). Obviously, the cranial part of the bone is both morphologically and by chemical composition more suitable than the caudal one for the performance of mechanical functions.

We did not investigate in detail the chemistry of the calcium salt components of pig bone. We, therefore, rely on the pertinent literary data in supposing that the microhardness of the bone depends not only on the actual quantity of bone minerals, but also on alterations in their chemical composition and crystalline structure.

Summary

The correlation between ash content and microhardness was studied on extremity bones (humerus, radius, ulna, metacarpus, femur, tibia, metatarsus) of 16 domestic pigs of different ages and one wild boar. The structure of the bones was also examined.

The microhardness of the bones showed a direct correlation with the degree of osseous mineralization. Bone ash content in per cent and microhardness differed between sectors of the femoral diaphysis, having been greatest in its central part, and greater in the compact cranial than in the spongiform caudal part of the bone. The degree of osseous mineralization varied between the different extremity bones, and the average microhardness values of the latter varied accordingly. Ash content and microhardness both tended to increase with progressing age.

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ULTRASTRUCTURAL CUTANEOUS CHANGES IN ZINC-DEPLETED PIGS

By

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The dietary deficiency of zinc affects the entire organism through its adverse metabolic effects. In the pig, the most conspicuous sign of zinc depletion is the development of para- and hyperkeratotic skin lesions over the entire integument.

It has been shown that about 20% of the organism's total zinc reserve is present in the skin (GREAVES, 1970). The cutaneous zinc concentration is 13—21 ppm, and the zinc unevenly distributed in the skin layers. Histochemical studies have shown that the bulk of cutaneous zinc is to be found in the stratum germinativum of the epidermis, and in the epithelium of hair follicles and sweat glands.

Dietary deficiency of zinc accounts for a decrease of the cutaneous zinc concentration and, consequently, for reduced incorporation of thymidine into cellular DNA (IM et al., 1975), as well as retardation of DNA, RNA and protein synthesis (PORIES and STRAIN, 1970). Decrease of unsaturated fatty acids and alkaline phosphatase activity was also demonstrated. Evidence was presented that the total acid mucopolysaccharide content of the pig skin does not change in the condition of zinc deficiency, but its hyaluronic acid component is increased (THOMPSON et al., 1975). The vitamin A content of the blood plasma is decreased (STEVENSON and EARLE, 1956), suggesting a reduction of the cutaneous vitamin A content as well. The tyrosine, phenylalanine and methionine concentration of the hair drops significantly (HSU and ANTHONY, 1977).

The most characteristic morphological sign of zinc deficiency, para- and hyperkeratosis, is predominantly a skin lesion in the pig and ruminant, while in the rat and chicken it involves chiefly the oesophagus and in the squirrel monkey *(Lamini sciureus)* the tongue (TUCKER and SALMON, 1955; HURLEY, 1969; MACAPINLAC et al., 1967).

The skin lesions of zinc-depleted pigs have been extensively studied by light microscopy (KERNKAMP and FERRIN, 1953; GUPTA et al., 1972; SÜVEGES et al., 1974), but ultrastructural examinations have not yet been reported.

Electron microscopic studies of the skin of zinc-depleted pigs are described in this paper.

Material and methods

Six pigs naturally-diseased in clinical parakeratosis, and 12 pigs with experimentally-induced latent zinc deficiency were used.

The naturally-diseased pigs procured from small farmers developed the condition for lack of a mineral supplement (premix) for several months.

Experimental zinc deficiency was induced with a diet containing 18.3 g per kg calcium, 2.3% zinc-binding material in terms of phytic acid, and 29.8 ppm zinc. Skin samples were taken after a 120-day diet regime.

Skin specimens, the size of a pea, were excised under local anaesthesia from grossly-changed (upper third of thigh) and apparently intact (lower third of thigh) cutaneous areas of each pig.

About 2-mm-long by 1-mm-wide pieces of each specimen, cut vertically to the surface of the epidermis, were fixed first in 5% glutaraldehyde, then in 1% osmium tetroxide, dehydrated, and embedded in a synthetic resin. Semi-thin sections were stained with toluidine blue.

Zinc deficiency was checked by laboratory measurements. Pigs of both groups showed a considerably decreased zinc content in serum and frontal bone, and a moderate decrease of it in liver, kidney and pancreas. Serum alkaline phosphatase and pancreatic alpha-amylase activities were also decreased. Details of the laboratory analysis will be reported in another paper.

Results

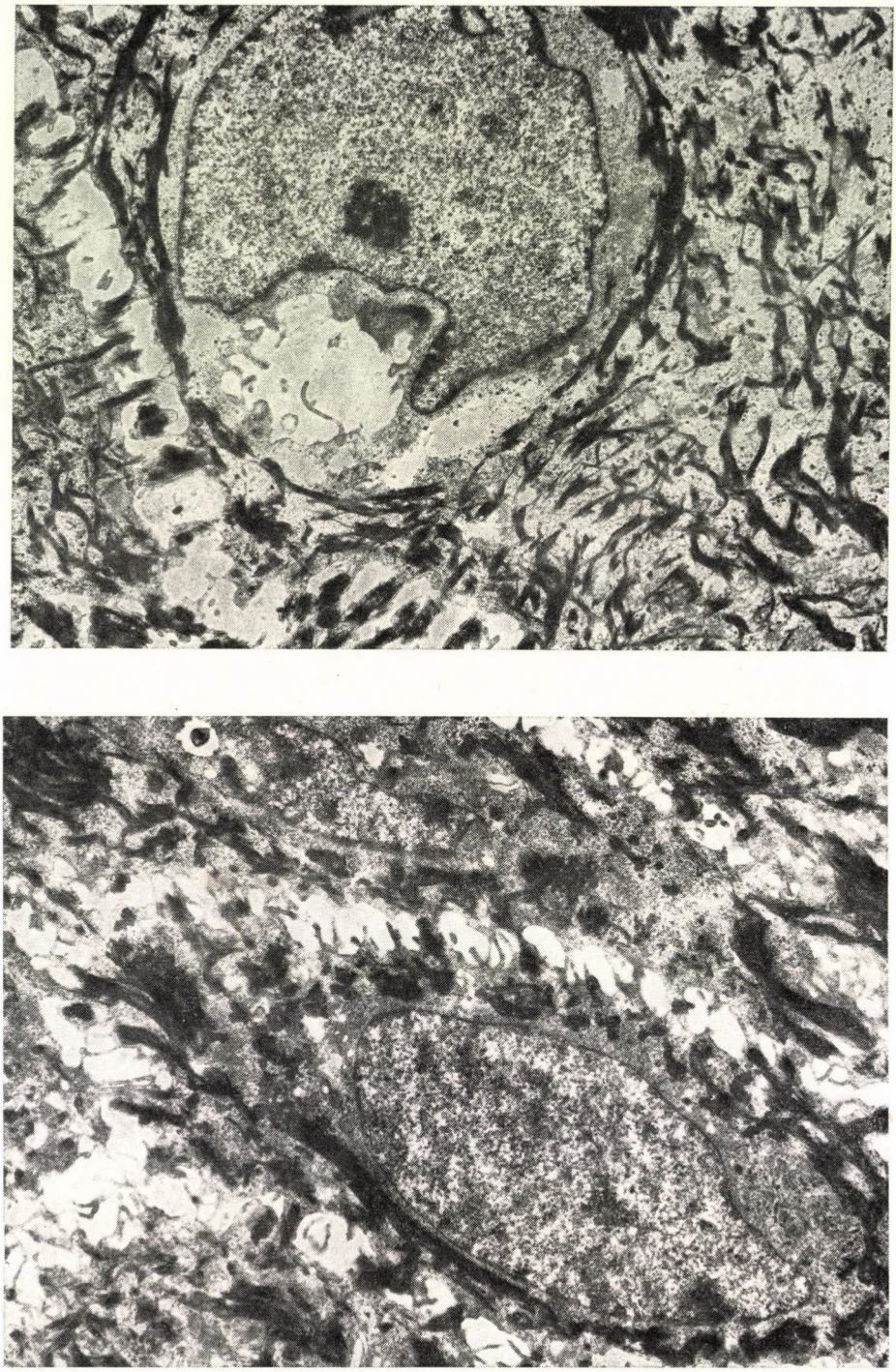
Light microscopic examination of semi-thin sections from severe cutaneous lesions of naturally diseased pigs revealed the presence of thick bundles of squamous horny epithelium in the epidermis. Abundantly vascularized papillae of the corium extended between the cornifying bundles. The superficial layer of the epidermis consisted of broad, hornified cell rows about to desquamate in places (Fig. 1).

Electron microscopy showed a slight dilatation of intercellular spaces in the stratum germinativum. There were slight retractions of the cytoplasm at the basal surface of the cells. Attachment to the basement membrane was by regular semi-desmosomes. The stratum germinativum cells were moderately electron-dense and rich in free ribosomes. The cytoplasmic bundles of tonofibrils were circularly arranged. In the stratum spinosum, the intercellular spaces were generally wide and the cells had a digit-like elongated shape. The tonofibrils lost contact with the desmosomes, owing to detachment of the latter in many places. The free desmosomes appeared in the intercellular space in the form of homogeneous electron-dense bodies of irregular shapes. The intermedial and medial zones of the desmosomes lost definition in most places (Figs 2 and 3). Some stratum spinosum cells had nuclei of irregular shape, owing to retractions. Many cells contained cytoplasmic vacuoles in juxtanuclear or other regions (Fig. 3). The tonofibrils surrounded the nucleus in a circular order, but were irregularly distributed in other cytoplasmic areas. The mitochondria varied in size, and were structurally more or less intact. Other stratum spinosum cells showed, chiefly in the superficial rows, a marked

Fig. 1. Abnormally wide, hornified detail of epidermis. Toluidine blue, ×270

condensation of tonofibrils, and the ribosomes increased in number (Fig. 4). The stratum granulosum was broad and contained several rows of cells; the nuclei were flat and oval-shaped, with contours rendered uneven by retractions. The cytoplasm contained many homogeneous, electron-dense, irregular keratohyaline granules (Fig. 5). MCG-granules were sporadically admixing with the keratohyaline granulation. The cells of the broad stratum corneum were in places long and narrow, elsewhere irregular in shape and of uneven width (Fig. 5). The fine structure of keratin was indefinite in some cells of the hornified layer. The intercellular space was wide and abnormal in places; no desmosomes could be seen. The superficial part of the stratum corneum contained some irregular, hornified epithelial cells, showing so-called spongy keratin structure (Fig. 6).

Electron microscopy revealed characteristic ultrastructural changes also in grossly intact-appearing cutaneous areas. Some epithelial cells of the stra-



Figs 2 and 3. Dilated intercellular space and loss of intercellular functions in the stratum spinosum. ×11 000

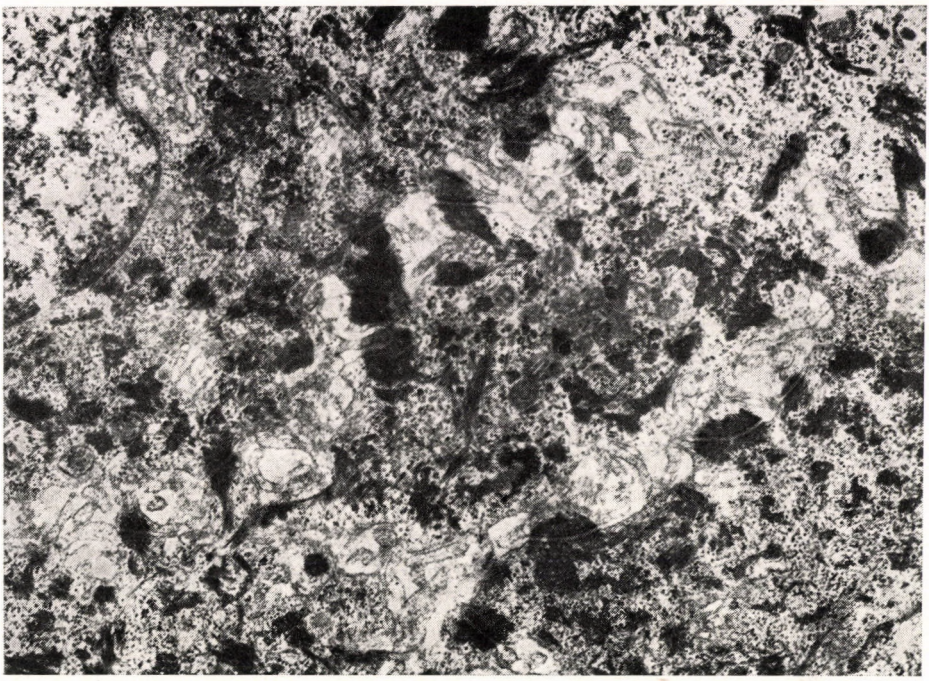


Fig. 4. Condensation of tonofibrils in stratum spinosum cells. ×23 800

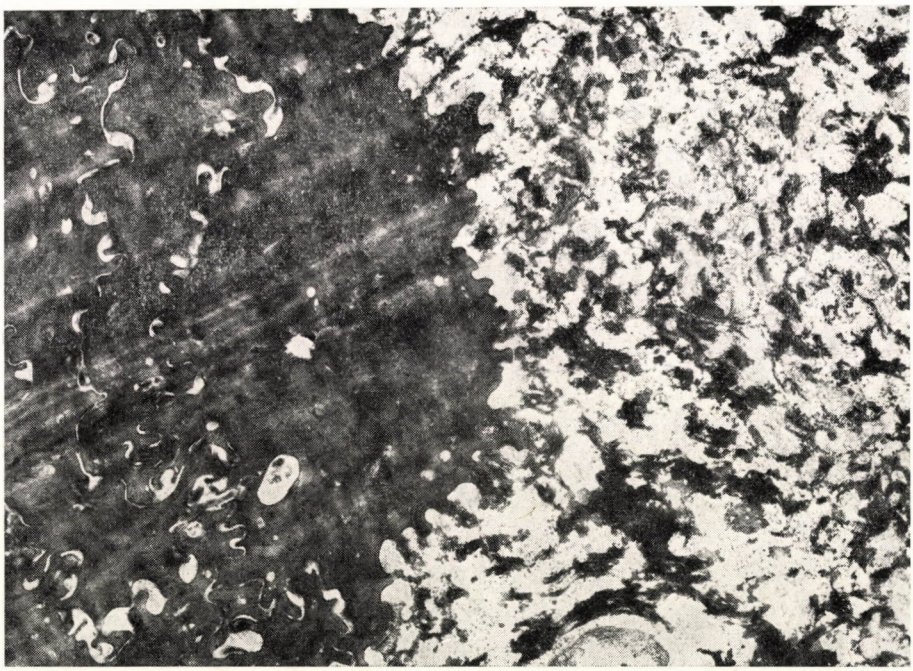


Fig. 5. Stratum granulosum and stratum corneum from the skin of a zinc-depleted pig. Note accumulation of keratohyalin granules in the granulosum cells and irregular, hornified corneum cells separated by lacunas. The cytoplasmic keratin structure has lost definition in places. ×10 200

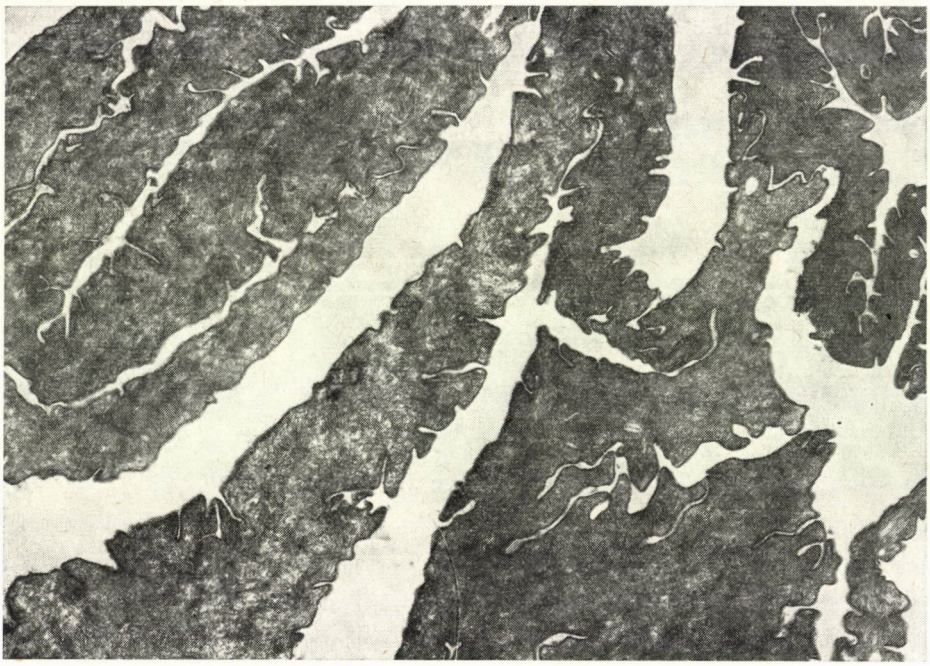


Fig. 6. Superficial row of the stratum corneum. The hornified, detaching epithelial cells have an irregular shape, and shaggy contrours. ×11 600

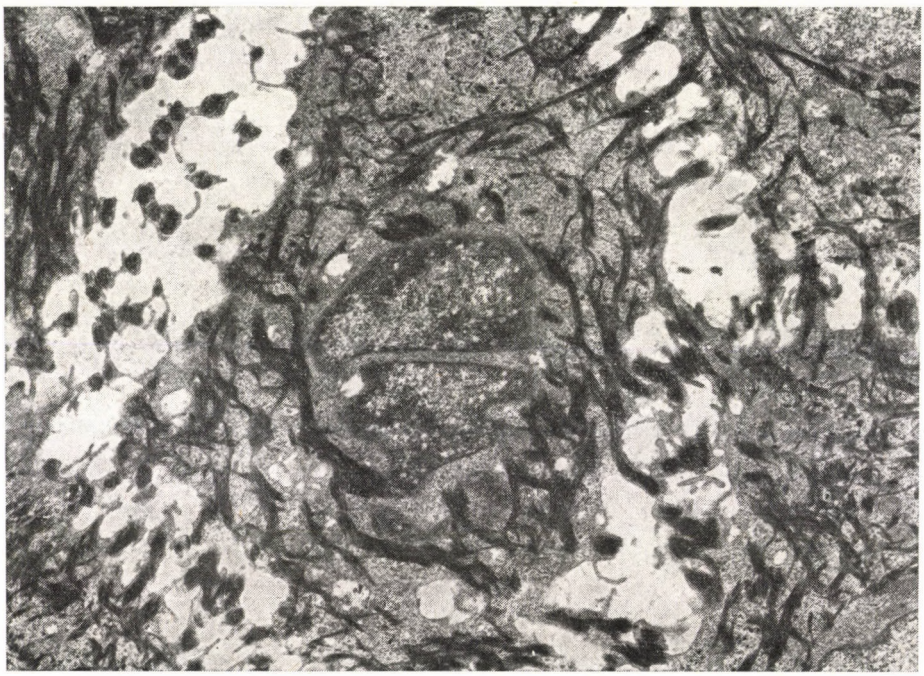


Fig. 7. The processes of the spinosum cells are damaged, and the desmosomes and tonofibrils lost connection owing to dilatation of the intercellular space. Note the homogeneous, electron-dense desmosomes. ×9700

tum germinativum extended long processes, and were detached from the basement membrane, which appeared indefinite. The intercellular spaces were generally dilated in the stratum germinativum. The epithelial cells of the stratum spinosum showed elongation of cytoplasmic processes, and loss of contact between tonofilaments and desmosomes. The fibrillar structure of the tonofilaments was indefinite in places. There were free desmosomes in the intercellular space (Fig. 7).

The pigs latently affected by experimentally induced dietary zinc depletion showed essentially the same ultrastructural changes as the clinically ill animals, but generally in a milder form.

Discussion

The skin of pigs naturally and experimentally diseased in zinc deficiency was examined for ultrastructural lesions. Specimens were prepared for electron microscopy from grossly altered and unaltered cutaneous areas.

Electron microscopic examination revealed that the gross para- and hyperkeratotic lesions had their origin in the stratum germinatium. The intercellular spaces between the cells of this layer were markedly dilated. The next layer, the stratum spinosum, was more severely involved. The tonofibrils of the stratum spinosum cells lost contact with the desmosomes, owing to detachment and injury of the latter. The tonofibrils themselves showed changes, such as loss of definition, or marked condensation of the fibrillar structure. Some stratum spinosum cells showed cytoplasmic vacuolation. Keratohyaline granule formation increased over normal in the stratum granulosum cells. The stratum corneum was broad; its cell rows were more numerous than normally and contained many hornified epithelial cells of irregular shapes. Broad lacunas often appeared in the intercellular spaces. Changed desmosomes were equally seen in the upper hornified and deeper cell rows. The keratine structure of some stratum corneum cells was indefinite.

The grossly intact-appearing skin of zinc-depleted pigs showed similar ultrastructural changes in the stratum germinativum and spinosum, but was lacking indications of hyper- and parakeratosis in the more superficial layers of the epidermis.

The ultrastructural picture suggest that the cutaneous cornification disturbance characteristic of zinc depletion begins with the pathological alteration of stratum germinativum and stratum spinosum cells. The main change is the injury of tonofibrils and desmosomes. The epithelial tonofibrils play and important role in the normal process of keratinization; some authors believe that the tonofibrils are responsible for the synthesis of the interfilamentary substance of keratin. Injury of the tonofibrils of pig epidermis by zinc defici-

ency presumably interferes with keratohyalin synthesis, and gives thereby rise to an abnormal cornification.

Presence of the characteristic initial ultrastructural changes leading to hyper- and parakeratosis in the grossly intact skin areas of zinc-depleted pigs can probably be utilized for an early diagnosis of the condition.

It should also be taken into consideration that pathological alterations of epithelial cells and intercellular junctions in the deeper layers of the cutaneous epidermis of zinc-depleted pigs presumably account also for depression of cutaneous resistance to mechanical effects. Thus, probably even physiological influences may interfere with normal cutaneous epithelial cell differentiation, and act thereby as an additional predisposing factor for the development of hyper- and parakeratotic changes. Grossly-visible cutaneous lesions do in fact appear in the skin areas most accessible to mechanical influences.

The ultrastructural cutaneous lesions of zinc-depleted pigs are essentially similar to those found by electron microscope in the epithelial coating of the tongue, palate, crop and plantar skin of zinc-depleted poultry and Japanese quail (WIGHT and DEWAR, 1977).

Summary

The epidermis of pigs naturally or experimentally diseased in zinc depletion was examined for ultrastructural changes. Electron microscopic examination showed that the hornification disturbance characteristic of the condition begins with cellular changes in the stratum granulosum and stratum spinosum. The main alteration is an injury of tonofibrils and desmosomes of the granulosum and spinosum cells. Damaged tonofibrils account for an abnormal keratohyalin synthesis, resulting in enhanced hornification. The keratotic alterations presumably act towards depression of cutaneous resistance to mechanical effects, so that even physiological influences may act as predisposing factors for the development of clinical zinc deficiency.

In grossly intact cutaneous areas of zinc-depleted pigs, the stratum granulosum and spinosum showed essentially the same ultrastructural changes as the hyperkeratotic regions, but the more superficial layers of the epidermis were not involved.

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INDEX

Virologia

MOCSÁRI, E.: Physicochemical Properties of Field and Cell Culture-Attenuated Strains of Swine Transmissible Gastroenteritis (TGE) Coronavirus 341

FEHÉRVÁRI, T., GLÁVITS, R. and RÁTZ, F.: Studies on the Egg-drop Syndrome 351

Mycologia

SÁNDOR, Gabriella, VÁNYI, A. and PETRI, Ágnes: Effect of Irradiation on the Viability and Toxin Production of Different Fungus Species 361

Parasitologia

MOLNÁR, K.: Cutaneous Sphaerosporosis of the Common Carp Fry 371

ŠLAIS, J.: Experimental Infection of Sheep and Pigs with *Echinococcus granulosus* (Batsch, 1786), and the Origin of Pouching in Hydatid Cysts 375

Immunologia

SÓLYOM, F., PERENLEI, L. and ROITH, J.: A Live Attenuated Virus Vaccine Against Sheep Pox 389

BODON, L., ZSÁK, L. and NAGY, Éva: Detection of Neutralizing Antibodies to Genuine Rabbit Adenovirus in Rabbit Antisera to Human Adenoviruses 399

Physiologia

MOUSTAFA, A. R. and MÉSZÁROS, I.: Interrelationship between the Total Protein Content of Bovine Seminal Plasma and Behaviour of the Spermatozoa after Freezing-and-Thawing 403

BOLDIZSÁR, H. and SIMON, F.: Effect of Zinc Sulphate Supplementation on Amino Acid Absorption from Pig Intestine. I. Intestinal Loop Tests 409

HARASZTI, J., SZENCI, O., SOLTI, L., FODOR, Eva and TÖRÖS, I.: Studies of the So-Called "partus syndrome" of Dairy Cows 419

RAFAI, P. and FODOR, Éva: Studies on Porcine Adrenocortical Function. I. Circadian Variations in, and Effect of ACTH Treatment on the Peripheral Plasma Cortisol Level 433

RAFAI, P. and FODOR, Éva: Studies on Porcine Adrenocortical Function. II. Effects of the Ambient Temperature and Restricted Feeding on the Peripheral Cortisol Level 443

Morphologia

SZILÁGYI, M., KOVÁCS, B. A. and PÁLFALVI, I.: Relationship between the Ash Content and Microscopic Hardness of Swine Bones 455

KAPP, P. and SIMON, F.: Ultrastructural Cutaneous Changes in Zinc-Depleted Pigs ... 463

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РЕЗЮМЕ

ФИЗИКО-ХИМИЧЕСКИЕ СВОЙСТВА ДИКИХ И В КЛЕТОЧНЫХ КУЛЬТУРАХ АТТЕНУИРОВАННЫХ ШТАММОВ КОРОНАВИРУСА СВИНОГО ТРАНСМИССИВНОГО ГАСТРОЭНТЕРИТА

Е. МОЧАРИ

Автором сравнивались некоторые физико-химические свойства трех диких и четырех в клеточных культурах аттенуированных штаммов вируса трансмиссивного гастроэнтерита свиней (ВТГЭ). Все штаммы были чувствительными к обработке хлороформом и 0,1%-ным деоксихолатом натрия и показывали слабую чувствительность к действию 0,1%-ного сапонина. Не обнаружено математически достоверной разницы в термической инактивации при 50 °С и стабильности к pH 3. 1 *M* $MgCl_2$ как правило ускорял термическую инактивацию, тогда как 1 *M* $MgSO_4$ незначительно усилил их термическую резистентность. Заметные разницы штаммов ВТГЭ в резистентности к действию 0,5%-ного трипсина не совпадали с их вирулентностью.

Автор приходит к заключению, согласно которому стабильность к воздействию разных физико-химических факторов и резистентность к протеолитическому энзиму является специфическим свойством штаммов ВТГЭ и не обуславливается ни количеством пассажей через клеточные культуры, ни степенью аттенуации.

ИЗУЧЕНИЕ ТАК НАЗ. «ЭГ ДРОП» СИНДРОМА

Т. ФЕХЕРВАРИ, Р. ГЛАВИЧ и Ф. РАЦ

В 1977-м году в поголовье несушек авторами наблюдалась патологическая картина, характеризующаяся снижением продукции яиц и образованием ненормальной яичной скорлупы. Заболевание началось жидким поносом, к которому позже присоединились снижение продукции яиц, образование деформированных, с тонкой, плохо обызвествленной скорлупой яиц. Количество падежей во время болезни не повысилось, поедаемость, корма не ухудшилась. Заболевание продолжалось 2—4 недели, в некоторых случаях яичная продукция и позже колебалась. При вскрытии убитых и обескровленных кур патологоанатомических изменений не обнаружено. При патогистологическом изучении половых органов в проприи слизистой матки наблюдали воспаление, которое сначала характеризовалось отеком и перерождением желез, выделяющих известь, а позже — инфильтрацией мононуклеарными клетками и образованием лимфатических узелков. Последнее явление наблюдалось и в других органах. Выявленные иммуно-электронмикроскопическим методом в содержимом тонкого и слепых кишок частицы, принадлежащие в группу аденовирусов, оказались тожественными таковым в антигене BC 14 Intervet. От этого антигена в сывороточных парах от больных животных наблюдали повышение титра.

ДЕЙСТВИЕ ОБЛУЧЕНИЯ НА ЖИЗНЕСПОСОБНОСТЬ И ТОКСИН ПРОДУЦИРУЮЩУЮ МОЩНОСТЬ РАЗНЫХ ВИДОВ ГРИБКОВ

ГАБРИЕЛЛА ШАНДОР, А. ВАНИ и АГНЕШ ПЕТРИ

Пищевые продукты уже давно с успехом обезвреживаются облучением. На основании этого факта авторами изучена резистентность грибков и их токсинов, засоряющих корма к облучению.

В качестве модели использованы эмульсии спор грибка **F. culmorum**, продуцирующего интенсивно токсин Ф-2, **A. ochraceus**, продуцирующего охратоксин А и **P. urticae**, продуцирующего патулин соотвественно, которыми искусственно засорялась кукуруза. В эксперименте с токсином Ф-2 кукурузу обрабатывали температурой, лучами и температурой + лучами соответственно. В экспериментах же с суспенсией спор грибка Аспергиллус и Пенициллиум кукурузу только облучали. После определенного срока инкубации готовили высевы грибков и определяли их продукцию токсинов.

В образцах с температурной обработкой фузариумы уничтожились, аспергиллусы и пенициллиумы — выжили. После облучения способность размножения грибковых штаммов понизилась, но у выживших таковых токсинпродуцирующая способность усилилась. При совместной обработке лучами и температурой содержание токсина в образцах быстро резко повысилось и установилось на данном высоком уровне.

СФЕРОСПОРОЗ КОЖИ МАЛЬКОВ КАРПА

К. МОЛНАР

Сфероспороз кожи мальков карпа вызывается тем же видом — **Sphaerospora carassii Kudo, 1919**, который одновременно вызывает и сфероспороз жабров. Формы развития и споры этого вида из отряда **Myxosporidia** наблюдались в большом количестве в коже близ жабр у 13-месячных мальков зеркального карпа.

ОБРАЗОВАНИЕ ВЫПЯЧИВАНИЙ НА ЭХИНОКОККОЗНЫХ ПУЗЫРЯХ ПРИ ЭКСПЕРИМЕНТАЛЬНОЙ ИНВАЗИИ *ECHINOCOCCUS GRANULOSUS* (Batsch, 1786) ОВЕЦ И СВИНЕЙ

Я. ШЛАЙС

Овец и свиней из Московской области инвазировали яйцами эхинококка, полученными от доноров, искусственно инвазированных украинским свиным и узбекским овечьим штаммами эхинококка соответственно. Пузыри, образовавшиеся в подсвинках, инвазированных в 6-месячном возрасте этими штаммами, были стерильными. Фертильные пузыри получены только в одном случае при инвазии 2-месячного поросенка овечьим штаммом эхинококка. Тяжелые и массивные инвазии наступили в 6-месячных ягнятах от инвазии свиным штаммом. В последнем случае 26 месяцев после инвазии обнаружен один фертильный пузырь. Мултилобулярные пузыри развились у всех успешно инвазированных овец, главным образом при использовании свечьего эхинококкозного штамма. В то же время структура кист, развившихся у свиней, была симметрической формы и наблюдалась ей соответствующая тканевая реакция. В силу сильной чувствительности овец к эхинококкозному антигену вокруг пузырей образуется очень толстая, аваскулярная и некротическая фиброзная соединительная ткань, которой обусловливается и форма развивающейся кисты. Стенка последней образует мешковидные выпячивания, которые сначала плотно примыкают к активной эпителоидной реакции, позже их окружает некротизирующая зона. Таким образом тканевая реакция хозяина является причиной образования мултилокулярных кист в овцах.

ВАКЦИНА ПРОТИВ ОСПЫ ОВЕЦ ИЗ ЖИВОГО, АТТЕНУИРОВАННОГО ВИРУСА

Ф. ШОЙОМ, Л. ПЕРЕНЛЕИ и Я. РОЙТ

Приготовлена живая аттенуированная вакцина из естественного вирусного штамма оспы овец, изолированного в Алжире и аттенуированного путем серийного пассажа в клеточной культуре. Вакцина оказалась безвредной как для монгольских овец ландрасс, так и таковых мериносного типа; только многократные дозы вызывали незначительную кожную реакцию и временную гипертермию при внутрикожном введении. При подкожном способе применения не наблюдались ни локальные, ни общие побочные явления. Обработанные рекомендуемой дозой вакцины овцы (1000 инфекционных единиц на животное) вырабатывали прочный иммунитет, защищающий их против реинфекции вирулентными вирусными штаммами MH и DAB.

ВЫЯВЛЕНИЕ НЕЙТРАЛИЗИРУЮЩИХ СВОЙСТВЕННЫЙ КРОЛИЧНЫЙ АДЕНОВИРУС АНТИТЕЛ В АНТИСЫВОРОТКАХ ПРОТИВ ЧЕЛОВЕЧЬИХ АДЕНОВИРУСОВ, ПОЛУЧЕННЫХ ИЗ КРОЛИКОВ

Л. БОДОН, Л. ЖАК и ЕВА НАДЬ

При идентификации авторами первый раз в мире свойственного кроличьего аденовируса серологически. доказано, что изолированный из кроликов аденовирус не является приобретенным от человека или какого-то животного, а свойственным вирусом кролика. Убедительным доказательством этого явился тот факт, что антисыворотки против человечьих аденовирусов, полученных из лошади, не нейтрализовали свойственный кролику аденовирус. В антисыворотках против человечьих аденовирусов, полученных из кроликов, могут налицевствовать — в силу их зараженности — антитела против свойственных кроличь их аденовирусов. Причиной этого является тот факт, что в сыворотке, взятых для продукции антисывороток кроликов, могут налицевствовать свойственные аденовирусные антитела и они могут быть причиной заблуждения исследователя.

Сывороточный пул № VIII после изнурения кроличьим аденовирусом уже не нейтрализовал свойственный кроличий аденовирус, но не нейтрализовались им и аденовирусы человека типа 17, 22, 23 и 24, использованные для производства антисывороток. Объяснением этого является то, что типовые антисыворотки смесивались в пулы не в концентрированнома в разбавленном состоянии. При изнурении пул еще больше разбавился и не был в состоянии нейтрализовать.

Ни одна антисыворотка против человечьих аденовирусов, полученная из лошади, не содержала антител против кроличьего аденовирусного штамма RA-1. Таким образом обнаруживаемые в антисыворотках против человечьих аденовирусов из кроликов свойственные кроличьи аденовирусные антитела происходят от кроликов, зараженных свойственным кроличьим аденовирусом.

ВЗАИМОСВЯЗЬ МЕЖДУ ПОЛНЫМ СОДЕРЖАНИЕМ ПРОТЕИНА В БЫЧЬЕЙ ПЛАЗМЕ СЕМЕНИ И ПОВЕДЕНИЕМ ЖИВЧИКОВ ПОСЛЕ ЗАМОРОЖЕНИЯ И ПОСЛЕДУЮЩЕГО РАСТАЯНИЯ

И. А. Р. МУСТАФА и И. МЕСАРОШ

Авторы определяли содержание протеина в плазме семени и таковой крови у 69 быков производителей и сравнили с определенными параметрами и качеством семени. Определение осуществлялось при помощи метода авто-аналайзер.

На основании качества семени быков разбили в три группы: с отличным семенем (1), с хорошим, но от заморожения страдающим семенем (2), с плохим, заморожение не выдерживающим семенем (3). Среднее содержание протеина в плазме семени равнялось 5,8 $\pm$ 0,7, 7,6 $\pm$ 1,2 и 8,2 $\pm$ 1,01 мг/100 мл соответственно, а содержание его в плазме крови — 7,9 $\pm$ 1,17, 7,27 $\pm$ 1,3 и 9,16 $\pm$ 1,9 мг/100 мл соответственно.

В концентрации живчиков и соотношении таковых с энергичным движением и содержанием протеина в плазме семени и таковой крови наблюдалась отрицательная взаимосвязь как до, так и после заморожения. Высокая концентрация протеина в семени может быть причиной повышения пермеабельности оболочки живчиков и тем самым обусловить холодный шок и повреждение живчиков в процессе заморожения и растаяния.

ДЕЙСТВИЕ ПОДКОРМКИ СУЛЬФАТОМ ЦИНКА НА ВСАСЫВАНИЕ АМИНОКИСЛОТ У СВИНЬИ. I. ИССЛЕДОВАНИЯ НА ИЗОЛИРОВАННОЙ КИЩЕЧНОЙ ПЕТЛЕ

Х. БОЛДИЖАР и Ф. ШИМОН

В 37 экспериментах на 18 свиньях английской белой мясной породы, веса в 25—35 кг авторами изучено всасывание аминокислот у свиньи и действие разных доз подкормки сульфатом цинка на их всасывание. Эксперименты проводились в самоконтрольной системе, при помощи фистулы Тири—Велла на среднем отделе двенадцатиперстной кишки и подвздошной таковой. В изолированную кишечную петлю ввели основной раствор, содержащий аминокислоты L-аспарагиновая кислота, глицин, L-метионин, L-изолейцин, L-лизин, L-гистидин и L-аргинин в концентрации по 2000 ppm, к которому добавляли цинк в виде $ZnSO_4 \cdot 7H_2O$ в количестве 50, 150, 225, 1800 ppm. После 10 и 30 минут всасывания определяли уменьшение количества аминокислот, цинка и воды. Бóльшее количество аминокислот всосалось из подвздошной кишки, чем из двенадцатиперстной таковой. В рассасывании разных аминокислот наблюдалась существенная разница. Наличие малого количества сульфата цинка (50 ppm) существенно усилило всасывание всех аминокислот и оно проходило за исключением аргинина на одинаковом уровне. Всасывание воды было независимым от всасывания аминокислот. При повторении экспериментов на том же животном при повышении дозы цинка рассасывание глицина усилилось, нейтральных аминокислот — не изменилось, аргинина — ослабло.

ТАК НАЗ. СИНДРОМ ПАРТУС У КРУПНОГО РОГАТОГО СКОТА

Я. ХАРАСТИ О. СЕНЦИ, Л. ШОЛТИ, ЕВА ФОДОР и И. ТЕРЕШ

У 45 коров породы голштейн-фриз в марте-мае месяцах 1978 года изучались изменения показателей сывороточной трансаминазы глутаминовой и щевелевоуксусной кислот (СТГЩК) и картины белых кровяных клеток в крови в срок 8 дней до и 8 дней после отела. Среди них у 17 животных следили в течение 14—15 дней за изменениями содержания глюкокортикоидов в сыворотке. У 18 животных в течение 42-х дней после стела следили за активностью яичника на основании уровня прогестерона в сыворотке. В поголовье коров венгерской пестрой породы в феврале—апреле 1978 года у 104-х животных изучали формирование уровня холестерина в крови в течение 25 дней после отела. Ради доказательства отрицательных последствий недостатка моциона во время сухостоя и его значения в одном поголовье коров породы голштейн фриз животные ежедневно регулярно гуляли 2800—3000 м. Шесть месяцев спустя сравнили данные «брака», «сервис периода» и индекс оплодотворяемости с теми же данными предыдущего года, когда моциона не было.

Данные экспериментов, направленных на выявление причин синдрома партус, показывают следующее. Изменения показывают следующее. Изменения показарелей СТГЩК и холестерина в крови могут быть диагностически ценными уже в срок времени до отела. Показатели концентрации СТГЩК у коров возраста 5 лет и старше в момент отела и 10 дней после него математически достоверно больше, а холестерина математически достоверно больше, а холестерина математически достоверно меньше, чем у более молодых животных того же поголовья. Имеется положительная взаимосвязь между низкими показателями холестерина в перипартальный период и продолжительностью сервис периода. Нормальному эструсу после отела в срок между 4—6-й неделей предшествовали колебания уровня прогестерона в сыворотке порядка 2,18 $\pm$ 1,17 и 2,19 $\pm$ 1,31 нг/мл. У коров, которые в указанный срок показывали уровень прогестерона только в 1,08 $\pm$ 0,53 и 1,65 $\pm$ 1,24 нг/мл, опаздывал первый фертильный эструс. Наблюдения на 632, коровах, которые в период сухостоя подвергались регулярному моциону, показали, что среди них меньше погибло в перипуэрперальный период или вынуждено убито, сервис период у них был короче и их индекс оплодотворяемости был меньшим.

ИЗУЧЕНИЕ ФУНКЦИИ КОРЫ НАДПОЧЕЧНИКОВ. II. ДЕЙСТВИЕ ТЕМПЕРАТУРЫ ОКРУЖАЮШЕЙ СРЕДЫ И УРОВНЯ КОРМЛЕНИЯ НА КОНЦЕНТРАЦИЮ КОРТИЗОЛА В ПЕРИФЕРИЙНОЙ КРОВИ

П. РАФАИ и ЕВА ФОДОР

В 4-х экспериментах авторами изучалось действие микроклимата и кормления хуже оптимальных на функцию надпочечников у подсвинков F_1 крупная белая венгерская х голландская низменная. Содержание в течение 7 дней в холоде (5°С) и недокормление (440 кJMe/кг^{0,75}) явилось сильной нагрузкой для организма животных. Под ее действием математически достоверно и на продолжительное время повысился уровень кортизола в периферийной крови животных. Уровень кортизола у животных в оптимальных климатических условиях от ограничения в кормлении тоже повысился математически достоверным образом, но после 96 суток он понизился между крайние но характериные для свиньи пределы. Функция надпочечников у свиней, содержавшихся в температурных условиях на 15—17°С ниже и на 10—13 °С выше оптимального, математически достоверно усилилась. Усиление функции в этих случаях было только незначительным и переходным. Дальнейшие наблюдения необходимы для определения того, что данными изменения уровня кортизола можно ли характеризовать степень стрессового состояния, наступившего после определенной степени и характера нагрузки организма окружающей средой.

ИЗУЧЕНИЕ ФУНКЦИИ КОРЫ НАДПОЧЕЧНИКОВ. I. ДЕЙСТВИЕ ПОРЫ ДНЯ И ОБРАБОТКИ АДРЕНО-КОРТИКОТРОПНЫМ ГОРМОНОМ НА УРОВЕНЬ КОРТИЗОЛА В ПЕРИФЕРИЙНОЙ КРОВИ

П. РАФАИ и ЕВА ФОДОР

Авторами определялся уровень кортизола в периферийной крови 8 подсвинков F^1 венгерская крупная белая х голландская низменная в возрасте 50 и 150 дней соответственно два дня подряд в 6, 12, 18 и 24 часов радиоиммунным способом. В другом эксперименте по 5 подсвинков возрата 65 ней обработано внутримышечно 15 и 30 μг/кг соответственно человечьим адрено-кортикотропным гормоном (АКТГ) и в оптимальных условиях следили за формированием кортизола у них.

Уровень кортизола показывал характерные, порами дня обусловленные колебания, размах которых с возрастом сокращался. Активность коры надпочечника была наибольшей в 6 часов и к 18-у часу она снизилась до минимума. Разница среднего уровня кортизола в образцах крови, полученных в 6 и 18 часов, математически достоверна. Согласно исследованиям авторов уровень кортизола в периферийной крови подсвинков колеблется в пределах 16—44 μг/кг.

Человечий препарат АКТГ час после обработки повысил уровень кортизола на 455 и 641%-ов соответственно, который при меньшей дозе после 12 часов, а при большей таковой — после 18 часов понизился до исходного положения.

ВЗАИМОСВЯЗЬ МЕЖДУ СОДЕРЖАНИЕМ ЗОЛЫ И МИКРОСКОПИЧЕСКОЙ ТВЕРДОСТЬЮ КОСТЕЙ У СВИНЬИ

М. СИЛАДИ, А. Б. КОВАЧ и И. ПАЛФАЛВИ

Изучалась взаимосвязь между содержанием золы и микротвердостью костей конечности (плечевой, лучевой, локтевой костей, плюсны, бедренной, большеберцовой костей и пясти) у 116 свиней разного возраста и одного кабана. Изучена и структура костей.

Микротвердость костей показывает прямую корреляцию со степенью их минерализации. Разные отделы бедренной кости между диафизами содержали разный % золы и показывали разную микротвердость; наиболее твердой явилась средняя часть кости и более твердой краниальна компактная часть, чем ее кавдальная спонгиозная таковая. Степень минерализации разных костей конечности и их микротвердость сильно отличались друг от друга. Содержание золы в костях и их микротвердость с ворзрастом соразмерно расли.

УЛЬТРАСТРУКТУРАЛЬНЫЕ ИЗМЕНЕНИЯ В КОЖЕ СВИНЕЙ, СТРАДАЮШИХ НЕДОСТАТКОМ В ЦИНКЕ

П. КАПП и Ф. ШИМОН

Авторами изучались ультраструктуральные изменения в эпидермисе свиней, страдающих естественным и экспериментально вызванным недостатком цинка. Электронно-микроскопические исследования показали, что перебои в ороговении начинаются повреждением клеток слоев str. granulosum и str. spinosum. В клетках упомянутых слоев бросается в первую очередь в глаза повреждение тонофибриллов и десмозом. В силу этого образование кератогиалина направляется в абнормальную сторону, он становится ненормальным и образуется в избыточном количестве. В эпидермисе слабой резистентности, согласно предположению авторов, в наступлении гипер- и паракератоза могут играть роль и физиологические механические воздействия.

Наблюдения авторов показали, что в невооруженным глазом нормальной кажущейся коже паракератозных свиней тоже имеются характерные ультраструктуральные изменения.

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