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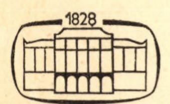

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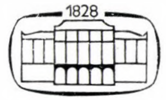

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INDEX

Tomus XVI

Fasciculus 1

Merétey, K., Várterész, V., Elekes, E.: Immunological Activity of Ribonucleoproteins. II. Immune Diffusion Studies of Ribonucleoproteins Extracted from Guinea Pig Liver 1
Merétey, K., Várterész, V., Kutas, V., Kocsár, L.: Immunological Activity of Ribonucleoproteins. III. Changes in Mammalian Ribonucleoproteins upon the Effect of Whole Body X-irradiation 11
Fodor, F., Kocsár, L., Lendvay, J., Merétey, K., Tapfer, D., Vedres, I.: Persistence of Human Gamma-globulin in Animals 19
Lázár, G.: Effect of Dibromomannitol on the Dextran-induced Increase of Local Capillary Permeability and on the Anaphylactoid Reaction 27
Bartha, T., Mérő, E., Szita, J.: Bacteriological Model Experiments on Radiosterilization of Catgut 31
Samir, M. A.: Studies on Peritoneal Exudate of Animals Experimentally Infected with *Toxoplasma gondii*. I. Effect of Heat on Activity 39
Lovas, B., Hollós, I.: Replication of Vaccinia Virus in the Embryonated Egg in the Presence of Methisazone. An Electron-microscopic Study 47
Elek, G., Földes, I., Vandra, E.: Effect of Dibromomannitol and Dibromodulcitol on Immunological and Microbiological Systems 63
Elek, G., Vandra, E., Földes, I.: Production of Antibodies against *Mycobacterium phlei* Phage 69
Horváth, S.: Transformation of Phage Resistance 77
Járai, M.: Biochemical Studies on *Streptomyces aureofaciens*. V. The Role of Cobalamins and Methionine in Methylation Reactions in Tetracycline Biosynthesis 85
Deák, S., Ádám, M. M.: Comparative Studies on the Biological Properties of *Escherichia coli* O124:K72(B17) Strains 97
Surján, J.: Studies on Swine Serum Immunoglobulins. I. Protein Fractions in Swine Serum 107

Fasciculus 2

Hollós, I.: Effect on Influenza Virus of a Modified Francis Inhibitor and Its Acetone-soluble Fraction. III. Experiments on the Mouse Lung 113
Gadó, I., Boromissza, É., Horváth, I.: Induction and Multiplication of λ-Phage. III. The Effect of Hydroxyurea on Heat Induction 123
Novák, E. K., Darvas, E., Zsolt, J.: Breakdown of Oligosaccharides by Some Afermentative Yeast Species 131
Füst, G., Keresztes, M.: Effect of Endotoxin on the Serum Level of Complement Components. II. Effect of Endotoxin on Dog Serum Complement Level *in vivo* and *in vitro* 135
Novák, E. K., Kevei, F., Oláh, B., Zsolt, J.: Investigations on the Oligosaccharide Decomposition by *Endomycopsis wickerhamii* van der Walt 149
Ralovich, B., Emődy, L.: Observations on Primary Bacteriaemia 153
Rauss, K., Kétyi, I., Máté, J., Kneffel, P., Ambó, G., Maróczi, J., Pusztai, S., Joó, I., Szendrei, L.: New Observations on Oral Vaccination against Dysentery 159

Váczi, L., Hankovszky, O., Hideg, K., Gergely, L., Hadházy, Gy.: Selective *in vitro* Antiviral Effect on Myxovirus Strains of Guanidine Derivatives 171
Csaba, B., Deseő, G., Kávai, M.: Distribution in the Organs of ^{131}I-Ovalbumin Antigen in Rat and Mouse Anaphylaxis 175
Dobozy, A., Hammer, H.: Some Properties of Alkaline Phosphatase in Bacillus Species 181
Koch, A., György, E.: Heart Glycosides in Poliovirus Host Cell Interaction. I. Effect of Digoxin and Digitoxin and Their Aglucons on One Step Growth Curves 189
György, E., Koch, A.: Heart Glycosides in Poliovirus Host Cell Interaction. II. Effect of the Time of Addition on Stimulatory or Inhibitory Action 197

Fasciculus 3

Réthy, L., Hegedüs, L.: Inhibition of the Development of Primary Antitoxic Immunity by Heterologous Antilymphocyte Serum 203
Csaba, B., Muszbek, L., Kassay, L.: The Effect of *Bordetella pertussis* Vaccine on the Histamine Metabolism of Rat Tissues 211
Rédai, I., Réthy, A., Rozgonyi, F.: Fatty Acids in *Staphylococcus aureus* and *Escherichia coli* Cultured *in vivo* 219
Rosztóczy, I.: Effect of Emetine on the Multiplication of Pseudorabies and Semliki Forest Viruses and on the Interferon Production by Cultured Cells 227
Raskó, I., Kerekes, E., Alföldi, L.: Properties of L-Serine Deaminase in *Salmonella typhimurium* and *Bacillus cereus* 237
Koch, A., György, E.: Heart Glycosides on Poliovirus Host Cell Interaction. III. Chemical Structure and Activity 245
Büki, K. G., Ambrus, G., Szabó, A.: Microbiological Decomposition of 17α-methyl-17β-hydroxy Steroids with Androstane Nucleus 253
Tömörkény, E., Albrecht, K., Ila, L.: Transformation of 4,5-Epoxysteroids with *Mycobacterium phlei.* I. Transformation under Aerobic Conditions 261
Füzi, M., Csukás, Zs.: New Selective Medium for the Isolation of *Clostridium perfringens* 273
Surján, J.: Studies on Swine Serum Immunoglobulins. II. Relationship between Immunoglobulins and Antibodies 279
Samir, M. A.: Studies on Peritoneal Exudate of Animals Experimentally Infected with *Toxoplasma gondii.* II. Susceptibility of Rats and Chickens 289
Csiszár, K.: Colicin Sensitivity and Colicinogenity of Type O124:K72(B17) *E. coli* Strains 295

Fasciculus 4

Ralovich, B., Vörös, S., Verténvi, A.: Antigenic Relationship between *Shigella sonnei* Phase II and *Enterobacter cloacae* 303
László, V. G., Kerekes, L.: Phage and Colicin Typing of *Shigella sonnei* 309
Tálas, M.: Interferon Stimulation and other Phenotypic Properties of Two Variants of Polyoma Virus 319
Lantos, J., Kiss, M., Lányi, B., Völgyesi, J.: Serological and Phage Typing of *Pseudomonas aeruginosa* Invading a Municipal Water Supply 333
Szentirmai, A., Horváth, I.: Properties of Acetohydroxyacid Synthetase in *Mycobacterium pellegrino* 337
Horváth, I., Ott, I., Gadó, I.: Induction and Multiplication of λ-Phage. IV. The Effect of Panfuran 349
Lányi, B.: Amino Acid Utilization by Serologically Grouped *Pseudomonas aeruginosa* Strains 357
Szilágyi, T., Csernyánszky, H., Imre, S., Went, M.: The Effect of Hypothermia on Allergic-type Skin Reactions 363
Weiszfeiler, J. G., Karasseva, V., Karczag, E.: Comparative Studies on the Taxonomic Relationship between *Mycobacterium abscessus* and *Mycobacterium borstelense.* Incidence of *Mycobacterium abscessus* in Monkeys 371
Szabó, I., Marton, M., Buti, I.: Intestinal Microflora of the Larvae of St. Mark's Fly. IV. Studies on the Intestinal Bacterial Flora of a Larva-Population 381

Acta microbiol. Acad. Sci. hung. **16,** *1—9, 1969*

IMMUNOLOGICAL ACTIVITY OF RIBONUCLEOPROTEINS

II. IMMUNE DIFFUSION STUDIES OF RIBONUCLEOPROTEINS EXTRACTED FROM GUINEA PIG LIVER

By

Katalin Merétey, V. Várterész and Erzsébet Elekes

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(Received February 16, 1968)

Summary. RNP extracted from guinea pig liver and the homologous immune serum form a multiplex antigen-antibody system. The number of antigen components demonstrable by immuno-electrophoresis ranges from 2 to 8. Some of the antigens are of nucleoprotein nature. The antigenic property of the nucleoprotein-like fraction isolated by electrophoresis also suggests this. The electrosyneretic preparations of *in vivo* ^{32}P-labelled antigen and immune serum gave positive autoradiographic pictures. The isolated fractions, containing solely nucleic acids and nucleotides, play no role in the serological reactions. The antigen components were identical with the antigens found in the subcellular fraction of the liver homogenate; their majority is both species- and organ-specific.

As confirmed by several data in literature, the ribonucleic acids have antigen properties [1, 2]. Assumedly, proteins also play a part in immunogeneity and the RNA acts only as a hapten. This is suggested also by the finding that the protein-bound nucleic acids (polyribonucleotides), nucleotides, nucleosides, elicit the production of hapten-specific antibodies [3, 4, 5]. The immunological studies of natural RNA-protein complexes were performed mainly on bacterial ribosomes [6, 7, 8, 9, 10]. The immune sera produced against the ribonucleoproteins (RNP) were assumed to contain antibodies owing to their complement-binding capacity, their activity in passive haemagglutination and their inhibitory effect on tumour transplantation [11, 12, 13]. Few immune diffusion data are only available on mammalian RNA extracted by chemical methods. Jankovich [14] demonstrated 5 various antigens in the ribonucleoprotein fraction of the rat liver.

As reported earlier, an immune serum has been produced in rabbits against the cytoplasmic RNP fraction isolated from guinea pig liver. The immune serum gave precipitation reaction with the RNP extract and the presence of nucleic acids could be demonstrated in the precipitate [15]. As an antigen, the extract proved to be heterogeneous.

The present paper reports on our immunodiffusion studies designed to elucidate the types and number of antigens in the RNP extract and the extent to which these antigens are identical with the antigen components in the liver homogenate.

Materials and methods

Preparation of the RNP extract. RNP was extracted from the pooled liver homogenates of 3 to 6 guinea pigs, according to PAIN and BUTLER (PB) [16] with some modifications [15]. Similar extracts were prepared from guinea pig spleen, rat liver and calf spleen.

The subcellular fractions of the guinea pig liver were obtained by preparative ultracentrifugation: mitochondrium (10,000 *g*), hyaloplasma (100,000 *g*), endoplasmic reticulum membrane (DOC-treated microsomal supernatant), ribosomes (105,000 *g*). The antigens were stored in the refrigerated or lyophilized state.

Preparation of immune sera. The immune sera were produced in chinchilla rabbits weighing 2 to 3 kg. Considering the weak antigenicity of the RNP extract, various adjuvants and immunization schedules were used. Complete or incomplete FREUND's adjuvant or $Al(OH)_3$ gel were given as adjuvants, intramuscularly and intravenously, respectively. The effect of RNase was eliminated by heparin [17]. Antigen injection into the spleen was also attempted. Administering of 5 mg of antigen with the same amount of complete FREUND's adjuvant at 15 day intervals six times proved to be the best for successful immunization. The rabbits were exsanguinated on the 10th to 14th days after the last injection.

Preparation of the ^{32}P-labelled extract and its autoradiographic study. Guinea pigs were given 2 mCi ^{32}P intraperitoneally. They were killed 12 hours after the injection and the RNP fraction from their liver was extracted. KODAK AR 50 stripping film was used for autoradiography. The slides were exposed for 14 to 21 days.

Agar. Veronal buffer pH 8.2 of 0.05 ionic strength and 1% Noble agar (DIFCO) were used.

Agar-electrophoresis. Electrophoresis was performed according to URIEL [18]. The antigen well was cut nearer to the negative pole, since the demonstrable components migrated towards the anode. Electrophoresis was carried out at 3 V/cm for 2 hours. The percentual distribution of the fractions in the stained preparations was determined by an ERI 10 unit.

Agar blocks $8.5 \times 6.0 \times 5$ cm in size were used for preparative electrophoresis. Fractions were recovered by freezing and thawing.

Electrosyneresis was adjusted according to BUSSARD [19]. There was a distance of 2 cm between the antigen and immune serum reservoirs.

Immunoelectrophoresis. SCHEIDEGGER's [20] micromethod was adopted. The comparative studies were performed according to OSSERMAN [21].

Gel diffusion studies. OUDIN's linear double diffusion method as modified by BACKHAUSZ [22] was applied. Three ml of 1% agar, dissolved in saline, were poured on 4×4 cm glass plates. The angular wells for the antigen and the antibody were cut at a distance of 6 mm from each other and filled with antigen or antibody mixed with agar. This arrangement allowed for satisfactory determination of the identical antigen components in the various extracts. The phenomenon when the precipitation line of the immune serum with extract "a" discontinued and did not deviate in the agar part corresponding to extract "b" was also regarded as identity reaction. To interpret the phenomenon we assume that both extract "a" and "b" contain identical antigen determinants, but these are so unfrequent on the molecules of extract "b" that no precipitate can develop.

Staining procedures. For protein staining, amido black was used. The nucleic acids were stained with pyronine, and were demonstrated by the gallocyanine chrome alum method. A 2.5% chrome alum solution containing 0.075% of gallocyanine was prepared according to LONAI [23] and the pH was adjusted to 1.0. The agar plates were stained for one hour and were then differentiated by tap water. A blue colour indicated the presence of nucleic acids.

Results

1. *Electrophoretic heterogeneity of the RNP extract in agar gel.* The extract yielded 4 fractions when subjected to agar electrophoresis (Fig. 1). Protein could be demonstrated at the site of application and in the slow fraction (a, b) migrating towards the positive pole. All the fractions proved to be positive on nucleic acid staining (a, b, c, d) (Fig. 1). Migration of the two rapid fractions

was compared to that of a preparation of di-penta nucleotides.* Since both fractions were localized over about the same area, they were taken to be of an oligonucleotide nature. Neither protein, nor nucleic acid could be demonstrated towards the negative pole.

2. *Reaction of the RNP extract and of its homologous antibody in agar gel.* Depending on the experimental series, more or less antigens could be demon-

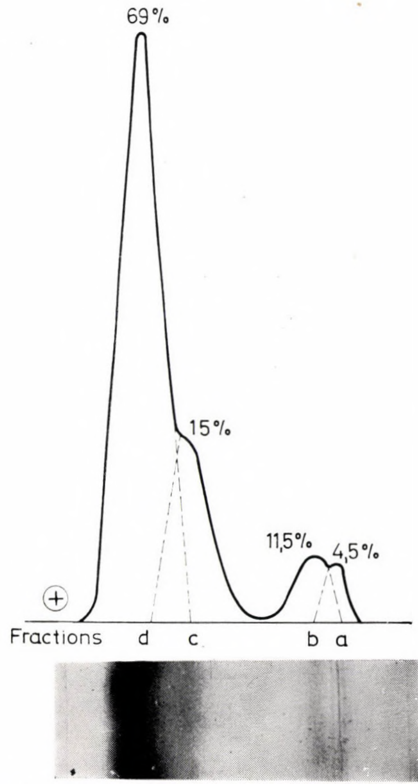

Fig. 1. RNP extract No. 35. Agar-electrophoretic picture on nucleic acid staining and the quantitative distribution of nucleic acids. *a:* Fraction persisting at site of application; *b:* slow fraction; *c* and *d:* rapid I and II fractions

strated in the RNP extract under the same preparation conditions. The number of the precipitation lines obtained by gel diffusion ranged from 2 to 5. Two to 8 antigen-antibody systems could be differentiated by immunoelectrophoresis (Fig. 2). Thus *e.g.* 5 components could be detected in extract No. 34 and 7 components in RNP extract No. 35 originating from another prepara-

* The preparations were kindly supplied by the Biochemical Institute, Debrecen. According to our analysis, they contained 7.3% of phosphorus and consisted of di-penta nucleotides.

tion. Serum protein impurities were not found among the demonstrated antigens.

Electrophoretic fractions were isolated from 6 different RNP extracts by preparative agar-electrophoresis. The separated fractions were characterized by their UV absorption spectra (Fig. 3).

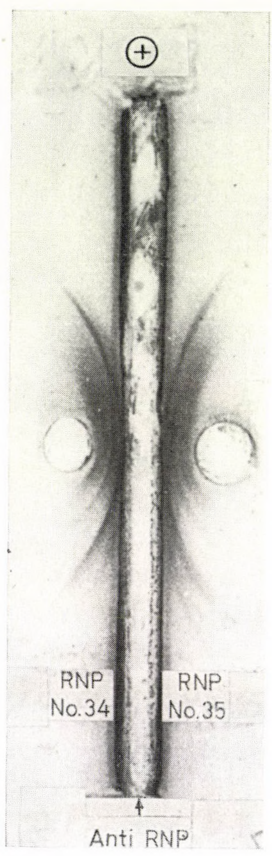

Fig. 2. RNP extract. Immunoelectrophoretic picture

Corresponding to the specific staining, the "b" and the "c+d" fractions gave nucleic acid and nucleotide type curves, respectively. At the antigen concentration used neither nucleic acids nor proteins could be detected either at the site of application (a) or in the non-staining agar layer (x) toward the cathode, though by immunoelectrophoresis one could detect antigens corresponding to this electrophoretic fraction. Serological activity of the fractions was controlled by electrosyneresis and immunoelectrophoresis (Table I). Only the slow fraction "b" migrating toward the anode has never failed to give

precipitation reaction. In certain extracts the "a" and the "x" fractions also reacted with the immune serum. The two rapid fractions ("c+d"), comprising a considerable part of the nucleic acids, did not precipitate at all. However, as confirmed by absorption studies, they did not inhibit the antigen-antibody reaction.

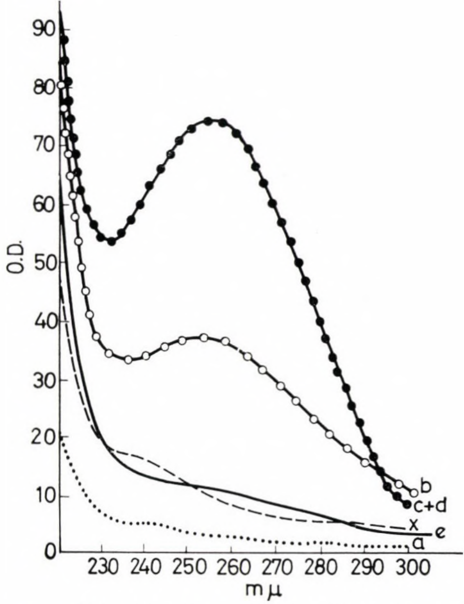

Fig. 3. Absorption spectrum of the fractions obtained by preparative agar-electrophoresis. *x:* agar towards the negative pole; *a:* fraction persisting at site of application; *b:* slow fraction; *c + d:* mixture of rapid fractions I and II; *e:* agar area towards the positive pole

The components of nucleoprotein nature and possessing antigenicity were studied by electrosyneresis. This allowed to demonstrate in the extract such antigens which by immunoelectrophoresis precipitated only from concentrated

Table I

Characterization of ribonucleoprotein fractions obtained by preparative agar electrophoresis

	Fractions*				
	x	*a*	*b*	(*c*+*d*)	*e*
Protein content	—	+	+	—	—
Nucleic acid content	—	±	+	++	—
Electrosyneresis	—	—	+	—	—
Immunoelectrophoresis	±	±	+	—	—
Precipitation inhibition	±	±	+	—	—

* The fractions are indicated as in Fig. 3.

extracts. The number of antigens so demonstrated, *i.e.* of those migrating towards the anode, ranged from 1 to 5. However, the gallocyanine or pyronine staining of the precipitates gave negative results. Neither seemed the precipitates to contain lipids. To elucidate the role of RNA, studies were performed

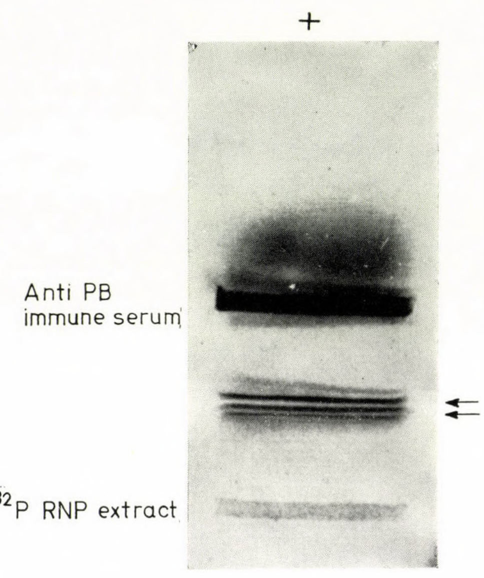

Fig. 4. Electrosyneresis of RNP extract ^{32}P labelled *in vivo*. The arrows indicate the positive precipitation lines obtained with autoradiography

with labelled antigens. The electrosyneretic preparations of antigen ^{32}P labelled *in vivo* gave a positive autoradiographic picture (Fig. 4). The immunoelectrophoretic preparations did not always display activity.

3. *Comparison of the RNP extract with subcellular liver fractions.* The subcellular fractions were compared with the RNP extract according to OSSERMANN [21]. The hyaloplasmic fraction contained all the antigen components found in the RNP extract (Fig. 5) and so did the mitochondrial

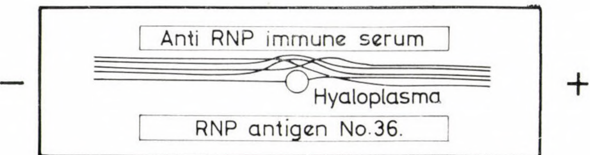

Fig. 5. RNP extract. Identity reaction with the hyaloplasmic fraction of guinea pig liver, performed according to OSSERMANN

fraction. On the other hand, a few antigens could only be demonstrated in the endoplasmic reticulum membrane. The ribosome fraction failed to display any serological activity with our immune serum.

4. *Organ- and species-specific antigens in the liver extract.* The RNP extract from guinea pig liver was compared with similar extracts from guinea pig spleen

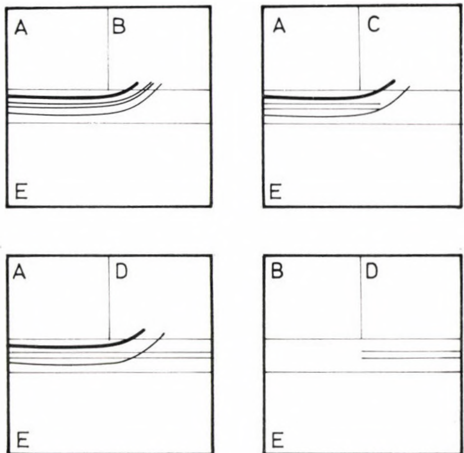

Fig. 6. RNP extract. Species-specificity by linear double diffusion method. *A:* RNP from guinea pig liver; *B:* RNP from calf spleen; *C:* RNP from rat liver; *D:* RNP from guinea pig spleen; *E:* anti-RNP serum

and calf and rat liver, by means of the linear double diffusion method (Fig. 6). The calf spleen extract did not react with the immune serum. The guinea pig spleen extract comprised two antigens which were common with those found in the liver RNP extract. RNP from rat liver failed to precipitate with the immune serum, but inhibited the deviation of these two precipitation lines.

Discussion

The present studies suggest that the RNP extract of the guinea pig liver isolated according to Pain and Butler comprises several antigens. The extract is heterogeneous also electrophoretically. Only the fraction containing nucleic acid and protein proved to be immunogenic. The exclusively nucleotide fractions were not immunogenic. It is questionable which of the antigens found in the extract are actual RNP antigens, and which of them are protein impurities left over after chemical preparation. No staining towards the negative pole (fraction x) could be seen on agar electrophoresis. Accordingly, the antigens migrating towards the negative pole, as observed by immuno-electrophoresis, are but minor impurities, while the curves found towards the posi-

tive pole could correspond to the electrophoretic localization of RNP. The fact, however, that the precipitates failed to stain contradicts this assumption. SCOTT [24] demonstrated that ion concentration strongly influences the results of pyronine staining. A low concentration, 0.05 *M* of NaCl, may also affect the staining of partially degraded or RNase-digested nucleotides, but washing in the usual 0.15 *M* NaCl and the drying create such an ion milieu which inhibits the binding of pyronine to the antigen components in the RNP extract. On the other hand, autoradiographic studies demonstrated a minute amount of precipitated RNP. When studied by electrosyneresis, two of the precipitation lines of the immune serum and of the ^{32}P labelled antigen did undoubtedly contain ^{32}P. In addition to nucleic acids, ^{32}P is known to incorporate into phospholipids. Staining failed to demonstrate any lipid in the extract. Accordingly, ^{32}P labelling suggests the presence of nucleic acids.

Owing to the many chemical procedures applied during preparation, the idea has arisen that the studies had been performed not with the native antigens in the cell but, in fact, with artificial antigens.

Antigen identity obtained in the different subcellular fractions suggests that no physical or chemical changes affecting antigenicity have occurred. It is difficult to interpret the negative reaction yielded by the ribosome fraction. The possibility arises that immunodiffusion studies performed in agar gel do not ensure optimum conditions for the immune precipitation of the ribosomes.

As shown by BARBU and PANIJEL [6, 10], RNA as an antigen is not strictly species-specific. That is why we tested the serological reactivity of RNP extracts of different origin. The extract used was not organ-specific, it contained two antigens common with those in the spleen extract. There was no antigenic relationship between the distant species. Still, the extract from guinea pig liver cannot be considered strictly species-specific since there was a so-called inhibitory type identity reaction between the two antigens of the guinea pig and rat liver extracts.

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IMMUNOLOGICAL ACTIVITY OF RIBONUCLEOPROTEINS

III. CHANGES IN MAMMALIAN RIBONUCLEOPROTEINS UPON THE EFFECT OF WHOLE BODY X-IRRADIATION

By

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Summary. The agar electrophoretic distribution of nucleic acids was different in the RNP extracts isolated from the liver of irradiated or normal guinea pigs. The alterations affected mainly the oligonucleotide fractions. According to immunodiffusion and immunoelectrophoretic studies no essential alteration occurred in the antigen components of the extract. The diffusion coefficients for the antigen components were 0.5×10^{-7} and 7.3×10^{-7} in the normal extracts, while they ranged from 0.7 to 9.9×10^{-7} in the irradiated ones. Both the normal and the irradiated RNP extracts had complement fixing activities. No difference was found in the RNase or trypsin digestibility of the extracts. Irradiation of guinea pigs did not affect the antigenicity of liver ribonucleoproteins.

A number of data prove that irradiation induces changes in the functional activity of nucleic acids, especially in the function of DNA. DNA isolated from the cells of heavily irradiated animals has altered physico-chemical properties [1, 2, 3, 4]. The altered structure may involve changes in antigenicity. The appearance of new antigen determinants in UV irradiated DNA has been described [5].

In the present study RNP extracts from the liver of normal (RNP) and irradiated guinea pigs (↯RNP) were compared by serological methods.

Materials and methods

RNP extract and immune serum were prepared as described previously [6].

Irradiation. Male guinea pigs weighing 350—400 g were whole body irradiated with 600 R (180 kV, 10 mA, 0.5 mm Cu filter, distance between focus and the middle of the body 50 cm, dose rate 39.1 R/min), 18 hours before killing and processing.

Immunodiffusion and immunoelectrophoresis. In addition to the methods described in a previous paper [6], angular diffusion [7] was used to determine the diffusion coefficients of the antigen components. The same immune serum and the dilutions of various extracts were measured into wells situated at angles of 90°. If the proportions are equivalent, the precipitation line forms at the site where the antigen and the antibody first meet. Once the direction of the precipitation line is known, on the basis of the equation $a/b = \sqrt{D_{ag}(D_{ab})}$ one may conclude upon the diffusion coefficient of the reacting antigen. The diffusion coefficient of the antibody (IgG) was taken for 3.96×10^{-7} cm²/sec. The distances between the precipitate and the reservoirs (a and b) were measured on magnified photos and on stained preparations.

Anticomplementary activity was studied according to KABAT and MAYER [8]. The RNP extracts were incubated with 100 $C'H_{50}$ guinea pig complement at 37 °C for 30 minutes. The complement contents of the mixtures and of the control mixed with saline were determined

spectrophotometrically by the haemolysis method. The anticomplementary activity of the normal and irradiated RNP extracts were related to an identical extinction (260 mμ).

Digestion. RNP antigen at a concentration of about 10 mg/ml was used. RNase digestion was performed at pH 7.0, in the presence of 1 mg/ml pancreatic RNase. Digestion with trypsin was done in a buffer solution of pH 8.0, in the presence of 0.5 mg/ml enzyme (lyophilized trypsin, Gedeon Richter Pharmaceutical Works, Budapest). After RNase digestion the undigested antigen components were separated by precipitation with 66% ethanol. Trypsin digestion was stopped by 0.1 *N* HCl (pH 6).

Statistical analysis of groups displaying about the same standard deviation was done with STUDENT's *t*-test.

Results

1. *Study of the RNP and ↯RNP extracts by agar electrophoresis.* Various RNP extracts prepared from irradiated and normal animals were subjected to agar-gel electrophoresis. According to quantitative evaluation, nucleic acid distribution in the irradiated nucleoprotein extracts differed from the normal (Table I). The component persisting at the site of application [9], consisting mainly of protein containing a low amount of nucleic acids, did not change. The quantity of the antigenic slow fraction was somewhat increased while that of the rapid oligonucleotide component I increased significantly. The quantity of the oligonucleotide fraction II decreased appreciably but the difference was statistically not significant.

Table I

Distribution of nucleic acids in normal and in vivo irradiated RNP antigen-series in agar-electrophoresis*

Fraction	Normal	Irradiated	p^+
Site of application	3.9 ± 1.49	3.8 ± 0.97	> 0.9
Slow fraction	13.5 ± 1.49	16.5 ± 6.10	
Rapid component I	14.3 ± 3.20	20.3 ± 4.30	0.02 < 0.05
Rapid component II	68.3 ± 4.23	59.3 ± 6.10	0.05 < 0.1

* Mean of relative percentages after staining with gallocyanine chrome alum, calculated on the basis of 7 normal and 5 irradiated antigen series.

+ Statistical significance according to *t*-test

2. *Immunodiffusion and immunoelectrophoresis of RNP and ↯RNP extracts.* No evaluable differences were observed between irradiated and normal antigens either by linear double diffusion or by immunoelectrophoresis. The antigen components of the various RNP extracts displayed slight quantitative differences. The differences observed could not be ascribed to irradiation. The

number of the demonstrated antigen components ranged from 2 to 8 both for normal and irradiated extracts originating from various preparations. These contained identical antigen components agreeing in electrophoretic mobility.

In absorption studies the immune serum produced against the irradiated extract failed to precipitate after absorption either by normal or by irradiated RNP extract. The same result was obtained on absorption with immune sera produced against the normal extract (Table II). In good agreement with the absorption studies the irradiated and normal antigen components displayed full identity by OSSERMANN's method.

Table II

Absorption of irradiated and normal RNP extracts

Immune serum	Antigen	
	RNP	↯ RNP
anti RNP	+	+
anti RNP absorbed by ↯RNP	—	—
anti ↯RNP	+	+
anti ↯RNP adsorbed by RNP	—	—

The diffusion coefficients of the antigen components of 4 normal and 3 irradiated RNP extracts were determined by the angular diffusion method. As seen from Table III, the diffusion coefficients of the irradiated antigen components were, as a rule, higher than those obtained for the antigen components of normal preparations, too.

Table III

Diffusion coefficients of the antigen components of irradiated and normal RNP extracts

Antigen	I*	II	III	IV	V	VI	VII
RNP_{32}		5.1+	3.5	2.5	1.5	0.5	
RNP_{35}	7.2	7.2	3.3	2.5	3.3	1.0	4.3
RNP_{36}	6.4	5.4	4.0	2.6	1.9		
RNP_{37}	7.3	6.1			1.6		
↯ RNP_{33}	7.2	5.6	4.7	2.2	2.3	0.9	
↯ RNP_{34}	6.9	5.8	3.2	3.3	2.5	1.6	
↯ RNP_{38}	9.9	7.2	4.8	2.5	2.0		

* Antigen components identified in the various extracts.
\+ $D \cdot 10^{-7}$ $cm^2 \cdot sec \cdot ^{-1}$

3. *Anticomplementary activity*. According to Table IV, the preparation yielded extracts with highly different anticomplementary activities. Although the average anticomplementary activity found for the irradiated extract differed from that of the normal one, the complement binding capacity of the extract from the irradiated animals was somewhat more marked though the increase was not significant statistically.

Table IV

Complement fixation by RNP antigens

Antigen	Number of tests	Bound $C'H_{50}$ units	$S_{\bar{x}}$	$\bar{x}$
RNP_{32}	19	22.9	7.54	
RNP_{35}	16	31.1	6.71	22.2
RNP_{36}	5	11.3	9.72	
RNP_{N}	12	23.5	9.25	
↯ RNP_{33}	17	20.2	8.22	
↯ RNP_{34}	16	29.6	5.11	34.3
↯ RNP_{I}	12	53.2	9.92	

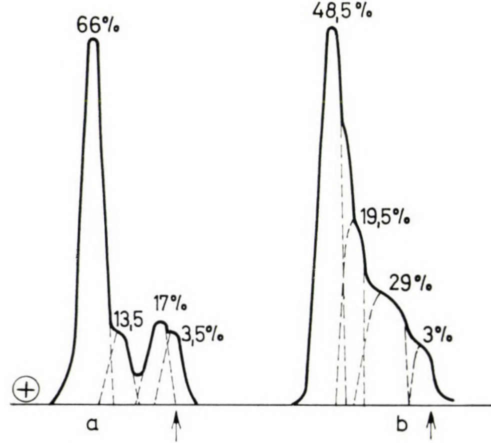

Fig. 1. Distribution of nucleic acids in normal (a) and trypsin-digested (b) RNP extract. Arrows show the site of application

4. *Digestion*. The RNP extracts were digested with trypsin at 37 °C for 24 hours, and the control (without enzyme) and digested extracts were compared. A characteristic change could be observed in the agar-electrophoretic preparations (Fig. 1). The mobility of the slow fraction increased and showed a wider zone. The relative amount of the fraction, the nearest to the anode,

decreased. Corresponding to the slow fractions, the situation of only one precipitation line changed in the immunoelectrophoretic preparations (Fig. 2).

The RNP extract was digested also with RNase at 37 °C for 24 hours. By the end of the digestion process the undigested components of the samples

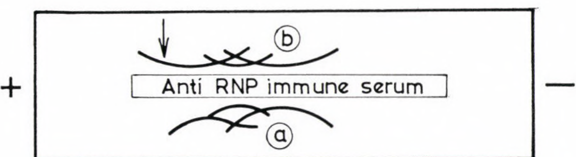

Fig. 2. Immunoelectrophoretic picture of trypsin-digested (b) and control (a) RNP extract

were precipitated with 66% alcohol. The agar electrophoretic preparations of the supernatants failed to stain either with gallocyanine or with amido black. The electrophoretic picture of the re-dissolved precipitate (Fig. 3) showed an unaltered slow fraction. The supernatants had no serological activity. In ab-

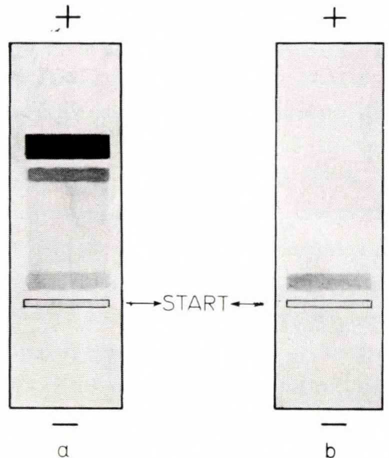

Fig. 3. Agar-electrophoretic distribution of nucleic acids in RNase-digested (b) and control (a) RNP extract

sorption and inhibition tests the highly concentrated supernatants sometimes reduced the number of the precipitation lines in the area toward the negative pole [10]. In serological reactions the re-dissolved precipitate behaved just as did the original extract.

The RNP and ↯RNP extracts showed no difference in digestion.

Discussion

It has been found that the quantity of the non-antigenic oligonucleotide fraction I and of the antigenic slow component was higher in the irradiated extracts. The various RNAs migrate at different electrophoretic mobility [11]. The ratio of RNAs of various origin might be different in the irradiated and normal extracts and this is reflected in the different distribution of nucleic acids in the agar-electrophoretic preparations. A radiation-induced change in the endogenous RNase level could also account for this difference. However, this assumption has not been confirmed by our experiments [12] according to which the endogenous RNase-induced spontaneous digestion of ^{32}P labelled normal and irradiated RNP extracts agreed. The increase in the slow component did not affect the antigen-antibody reactions. Slight quantitative differences were observed in the immunodiffusion and immunoelectrophoretic preparations. Owing to the experimental schedule, the extract was always prepared from different groups of guinea pigs. So, the differences observed may be explained only by quantitative differences arising in the course of preparation. The disappearance of an antigen determinant or the appearance of some new antigen determinant could not be demonstrated.

On the basis of the diffusion coefficients, the molecular weight of the antigenic substances in the extract changed between about 20 000 and a few millions. The diffusion coefficients were somewhat higher in the irradiated extracts. This must have been exclusively due to differences in preparation. On the other hand, one cannot exclude that the physico-chemical structure of the irradiated RNP extracts actually differs from that of the normal ones.

According to YACHNIN [13, 14, 15, 16, 17] some polynucleotides (poly I and poly G) have anticomplementary activity, while the natural nucleic acids do not display such a character. The fact that our RNP extract inhibited the activity of guinea pig complement contradicts these studies. On the other hand, we did not use phenol for extraction but acidic precipitation and heat denaturing which might have affected the secondary structure of the components in the extract that some of them became anticomplementary. It cannot be excluded that heat-denatured tissue protein impurities were also responsible for the anticomplementary effect. The anticomplementary activity of irradiated and normal extracts was almost identical, a high value could be observed only in one of the irradiated extracts.

HARRINGTON [3] demonstrated increased nuclease and alkaline phosphatase sensitivity in DNA isolated from irradiated lymphoblasts. The irradiated and control RNP extracts could be digested with both trypsin and RNase.

Irradiation of the animals did not appreciably affect either the antigenicity or the sensitivity to enzymes of the liver ribonucleoproteins. The differences found in the diffusion coefficients and in the electrophoretic mobility, varying

from case to case, could have been due to the various preparation methods just as well as to the radiation effect itself.

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PERSISTENCE OF HUMAN GAMMA-GLOBULIN IN ANIMALS

By

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Summary. Human heterologous gamma-globulin showed no fragmentation in the guinea pig during 2 to 3 weeks. For two weeks after the administration of human gamma-globulin labelled with iodine isotopes the highest specific activity was found in the blood. High values were demonstrated in the liver, kidneys and lungs. Serum antibodies against human gamma-globulin (0.125 g protein/kg body weight) appeared on the second week and persisted at a high level even three months after immunization.

Human gamma-globulin is widely applied in the prophylaxis of some infectious diseases. In Hungary, it is given to persons living in the environment of infectious hepatitis patients and to infants exposed to nosocomial staphylococcal infections. In some cases it is also used for the prevention or mitigation of measles.

Gamma-globulin prophylaxis was first recommended and used by Stokes and others. In Hungary Csapó *et al.*, Solt, Stoll, Losonczy and others reported on the results of mass inoculations [2, 7, 10, 11, 12, 23, 33, 37, 38].

In the opinion of Csapó *et al.* human gamma-globulin (HGG) induces both active and passive immunity against infectious hepatitis [11]. Other authors reject this opinion or regard HGG as an agent exerting a very doubtful active immunizing effect [8, 21].

HGG is produced from the blood of donors or from placental blood. As it is prepared from pooled blood samples taken from 400 to 800 or more individuals per batch, it is safe and, apart from a few sporadic local reactions, causes no postimmunization complications. Allergic reactions after HGG administration are rarely encountered [8]. HGG is usually given by deep intramuscular injection.

In the present study we investigated the appearance, level and persistence of HGG in the circulation, its deposition in the organs and the immunogenic effect of various doses of heterologous HGG.

The metabolism of macroglobulins was studied by several authors [1, 4—6, 31, 39, 40]. In the present experiments, similarly to other investigations using different protein antigens [3, 9, 13, 14, 16, 17, 19, 20, 24, 25, 27, 28, 30,

31, 34], the animals were immunized with a heterologous protein, ethanol-fractionated HGG.

Materials and methods

Animals. The guinea pig was chosen, partly because it is easy to handle and suitable for immunological experiments, and partly because its natural globulin and gamma-globulin level is fairly low.

There are few data for the protein composition of guinea pig serum [18, 35]. To establish the normal values, two groups of 14 guinea pigs each were examined for protein content and percentage distribution of serum protein components (Table I). Total protein was determined by the micro-Kjeldahl method and by parallel specific gravity measurements using the Phillips—van Slike method. Separation and quantitative determination of serum proteins were performed by paper electrophoresis [15].

Healthy female, non-pregnant guinea pigs weighing 180 to 260 g, were distributed into 2 main groups.

Group I. Thirty animals were divided into 3 subgroups. Each of them in the appropriate subgroup received 100, 50 or 25 mg (0.500, 0.250 or 0.125 g protein/kg body weight) of "cold" (unlabelled) HGG. Blood samples were taken by intracardiac puncture. Serum proteins with special regard to gamma-globulin were determined by paper electrophoresis. The heterologous HGG antigen and antibodies produced against it were determined by immunological methods as follows.

(a) Determination of HGG level in guinea pig serum was performed by linear immune diffusion using horse serum reacting with human immune globulins as described by OUDIN [29]. The HGG content of test sera was determined in comparison to standard dilutions of HGG.

(b) For determining the anti-HGG titre in guinea pig serum, Rh positive (D positive) washed human erythrocytes were sensitized with human anti-D blood grouping serum. The suspension was incubated at 37 °C for 1 hour, then the erythrocytes were washed three times in saline, resuspended to 2% concentration and added to serial serum dilutions prepared in TAKÁTSY's microtitrator. After 1 hour incubation at 37 °C the highest serum dilutions agglutinating the sensitized erythrocytes were determined. The results are presented in Figs 1 and 2.

Group II. ^{131}I-HHG was given to 10, ^{125}I-HGG to another 10 animals.

Human gamma globulin (commercial preparation, Institute for Serobacteriological Production and Research, Human, Budapest) was labelled with ^{131}I and ^{125}I by using the method of VEALL, PEARSON and HANLEY [43] and MCFARLANE [26]. The nativity of labelled HGG was checked by agar electrophoresis and immunoelectrophoresis according to OSSERMANN and SCHEIDEGGER. All preparations used in the experiments retained their nativity.

Table I

Protein components of guinea pig serum

No. of animals	Albumin	Globulins, relative per cent			
		$alpha_1$	$alpha_2$	beta	gamma
14	56—64	2—3	11—17	10—12	11—16
14	58—64	1.5—2.8	11.3—17.5	10.2—12.2	11.1—16.5

Total protein, 4.0—5.5 and 4.1—6.1 g per 100 ml

Results

After injecting large doses of HGG (0.500 g protein/kg) into guinea pigs the gamma-globulin serum level (total protein migrating on the paper strip at the site of gamma-globulins = the animal's own gamma-globulin + the in-

jected heterologous gamma-globulin) reached a maximum value after 3 days. This level decreased slowly in 3 to 6 weeks to a minimum still higher than the normal level. Subsequently a new rise due to specific antibody production was observed. This complex gamma-globulin remained at high levels (40 to 45 rel%) and could be detected after 8 to 10 weeks even by paper electrophoresis, a less sensitive method.

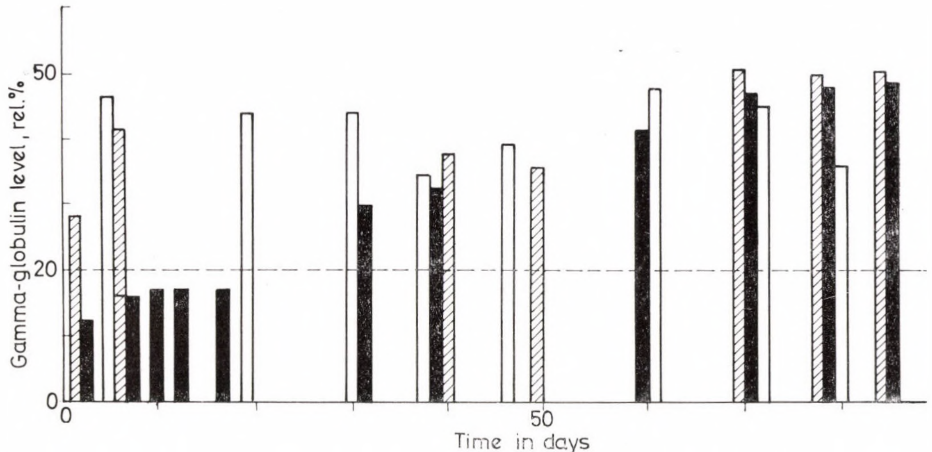

Fig. 1. Gamma-globulin level in guinea pig serum after the administration of HGG in doses o 0.500 g protein/kg (empty columns), 0.250 g protein/kg (shaded columns) and 0.125 g protein/kg (black columns)

When small protein doses (0.125 g/kg) were given, the rise in globulin fractions could not be shown with paper electrophoresis in the first weeks. From the 3rd to 4th week onward the gamma-globulin level increased significantly and remained at a level more than double the normal still after 10 to 11 weeks.

The results of immunological experiments with "cold" (not labelled) gamma-globulin using the linear immune diffusion technique and passive haemagglutination can be summarized as follows.

1. According to a typical experiment the disappearance of gamma-globulin given in large doses (0.500 g protein/kg) occurred in three phases. The half-life of the antigen was approximately 5 days in phase 1, 23 to 25 days in phase 2, and 4 to 5 days in phase 3.

2. When the antigen was injected in medium doses (0.250 g protein/kg), a two-phase clearance was observed. The half-life was approximately 4 days in phase 1 and 6 days in phase 2.

3. After the administration of small doses (0.125 g protein/kg), the antigen disappeared in two phases characterized by approximately 4 and 1 day half-lives.

Time course of antibody production. 1. Antibody production started 50 days after the administration of large doses and showed a rising tendency even on the 87th day.

2. Medium doses gave rise to detectable antibodies on the 20th day; maximum levels were reached between the 45th and 62nd days.

Fig. 2. Serum level, HGG antigen and antibodies produced against it in guinea pigs. HGG doses: 0.500 g protein/kg (dotted line), 0.250 g protein/kg (dashed line) and 0.125 g protein/kg (solid line)

3. Small doses started antibody production on the 10th day. Maximum levels had been reached by the 30th day and were maintained almost without decline even at the 80th day.

Accordingly, an asssociation was demonstrated between the antigen dose and the appearance of specific immune response. Human gamma-globulin persisted without alteration in the animals for 18 days. Immunoelectrophoresis of guinea pig sera taken at various intervals showed no evidence of fragmentation.

Radioactivity measurements. Differences in the gamma-globulin level in individual animals after injecting labelled HGG were determined by whole body activity measurement. The correction factors, which should be considered

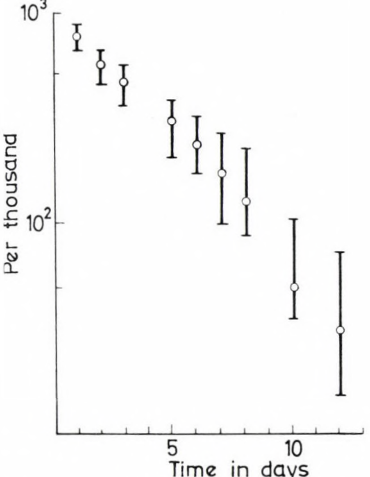

Fig. 3. Time course of ^{131}I-HGG activity in the blood of guinea pigs

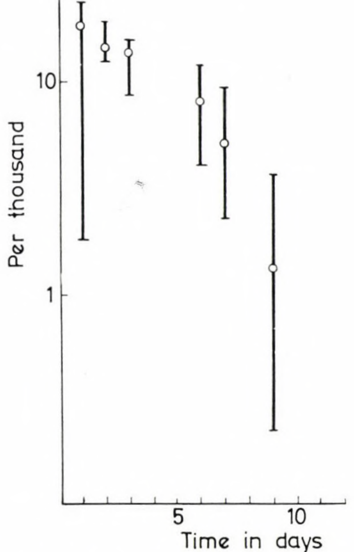

Fig. 4. Retention of ^{131}I in guinea pigs

in such types of experiment for all parameters of the animals in order to correlate the results for an equal level of radioactivity administered, were established for each guinea pig.

Blood and organs were examined by means of an energy selective counter supplied with a well-type NaI(Tl) detector. The paper electrophoretic examination of ^{131}I-gamma-globulin was checked by radioactivity measurement prior to administration to the animals.

It has been considered important to determine radioactivity in the blood originating from ^{131}I-HGG, since significant differences may in this respect exist between individual animals.

During the first 5 to 6 days the activity in 1 ml blood was 1% of the administered amount, a finding indicating a durable gamma-globulin exposition. Differences attributable to administration conditions had been equalized by the 6th day, but later, in consequence of the rapid clearance of the antigen, they again became considerable.

Subsequent examinations were conducted only in animals showing no significant differences in whole body radioactivity after receiving labelled HGG. After 14 days, 2 to 6-fold differences existed in the retention of the antigen. This finding means that animals with identical parameters may exhibit 4 to 70-fold differences in the radioactivity of their blood (Fig. 3). Each point in Fig. 3 represents the average of more than 5 individual determinations.

From the ^{131}I retention curve the degree of excretion may be estimated. This curve, similarly to that representing the specific activity in blood, shows a break at about the 4th to 6th day. Each point in Fig. 4 represents the average of more than 10 individual determinations.

Evaluation of radiometric measurements in organs was limited by the small number of samples. Specific activity was highest in the liver; the lungs and kidneys also showed high values. Within 14 days higher activity was measured in 1 ml blood than in any of the organs.

Discussion

The distribution of HGG in the organs of the guinea pig, changes in the serum globulin level, dynamics of excretion and secondary body responses have been examined. After intramuscular administration of normal and ^{131}I or ^{125}I-labelled HGG the results were evaluated by immunobiological, immunochemical, paper electrophoretic and radioactivity examinations. HGG given in large doses (0.500 g protein/kg) showed no fragmentation in guinea pigs for an average of 18 days and its excretion lasted for 6 weeks. HGG was excreted within 2 weeks when given in smaller doses (0.125 g protein/kg).

Specific antibodies against 0.500 g protein/kg HGG appeared after 6 weeks. Doses of 0.125 g protein/kg induced antibody production from the 2nd week onward; high antibody levels were shown even 12 weeks after immunization.

Iodine-labelled HGG showed 1% of the administered activity in 1 ml blood on the 1st week. This finding indicates a durable gamma-globulin exposition. Two weeks after administration, blood activity decreased to very low levels.

Among the organs the highest activity was exhibited by the liver. High activity was shown also by the lung and kidneys. In 2 weeks specific activity was higher in the blood than in any of the examined organs. Within this interval the lymph nodes showed a tendency to accumulate increasing amounts of radioactivity.

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EFFECT OF DIBROMOMANNITOL ON THE DEXTRAN-INDUCED INCREASE OF LOCAL CAPILLARY PERMEABILITY AND ON THE ANAPHYLACTOID REACTION

By

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Summary. Pretreatment of rats with dibromomannitol considerably suppressed the increase of capillary permeability elicited by HALPERN and BRIOT's method and also the dextran-induced anaphylactoid reaction. Considering the decisive role of histamine and serotonine release in the dextran-induced anaphylactoid reaction of the rat and their supposed role in the pathogenesis of the local Shwartzman phenomenon, suppression of the Shwartzman reaction by nitrogen mustard and its derivative dibromomannitol might be due not only to their leukopenic action, but also to the altered reactivity of the tissues.

Suppression of the Shwartzman reaction by leukopenia inducing agents, such as nitrogen mustard, benzene or X-rays, was described by BECKER [1] who attributed the effect primarily to rendering the endothelial cells "anergic" to endotoxin. As stated by BECKER "It is postulated that the mechanism of suppression by these agents is exerted through their specific but common suppressive action on the reticuloendothelial system, primarily the vascular endothelium. These endothelial cells being rendered anergic are not able to react to the active principles in a way that otherwise would be self-destructive." This hypothesis, based on the direct "tissular" action of the said agents was rejected and the suppressive action was ascribed to the leukopenia.

Studies by ANTOPOL and CHRYSSANTHOU [2, 3] then again drew attention to the role of tissular factors in the Shwartzman phenomenon, as they suggested that the release of biogenic amines played an important pathogenetic part. With these facts in mind, we have examined the influence on tissue reactivity of the leukopenia-inducing nitrogen mustard derivative dibromomannitol.

Materials and methods

Male albino rats of the R Amsterdam strain, 180—220 g in weight, fed a standard laboratory diet, were used throughout. The animals were treated intravenously with 4 mg/100 g dibromomannitol (Degranol) on two subsequent days. The experiment was started after 48 hours.

The dextran-induced increase of capillary permeability was examined by HALPERN and BRIOT's method [4] on 10 dibromomannitol treated and 10 untreated control rats. Following the intravenous administration of 0.5 ml/100 g of a 1% Evans blue solution 3 μg of dextran (GLAXO; molecular weight 130,000) was injected intradermally into the depilated dorsal skin of the animals. Thirty minutes later the rats were killed.

Further 10 animals were used to examine the ability to anaphylactoid reaction. A 30 mg/100 g dose of dextran was administered intraperitoneally and the extent of the ensuing anaphylactoid reaction was assessed by determining the water content of the hind legs. Two hours after the injection of dextran the animals were killed and their hind legs were weighed immediately and after dehydration in a 56 °C incubator for one week. The water content was related to 1 g dry material.

Evaluation of the results was done by STUDENT's *t*- test.

Results

Pretreatment with dibromomannitol resulted in a considerable decrease of the leukocyte count (Fig. 1).

In such rats the increase of capillary permeability elicited by HALPERN and BRIOT's method was markedly suppressed. The control rats exhibited an intensive blue area 1 cm in diameter, whereas in the treated rats the area was just slightly blue in colour (Fig. 2).

Dibromomannitol significantly inhibited the development of dextran oedema (Fig. 3).

Two hours after eliciting the anaphylactoid reaction the water content of the hind extremities of the control and treated animals was 1690 and 1160 mg, respectively, as related to 1 g dry material. Thus, protection against the anaphylactoid reaction was significant statistically.

Discussion

The role of leukocytes in the pathogenesis of the Shwartzman reaction has extensively been studied, but opinions concerning their role in this context are not uniform. STETSON and GOOD [5] attached a decisive importance to the metabolic change and consequent lactic acid increase due to leukocytic infiltration at the site of the preparative endotoxin injection, and suggested that the metabolic change enhanced the effect of intracellular proteolytic enzymes. In previous studies [6] we have succeeded in eliciting a local Shwartzman reaction by injecting endotoxin into rabbits and guinea pigs following cutaneous preparation with rabbit, guinea pig and rat peritoneal leukocyte suspension. The phenomenon has been attributed to the mononuclear cells, ability to transfer late type "endotoxin hypersensitivity". Following preparation of the rabbit's skin with leukocyte granules, THOMAS [7] elicited a haemorrhagic cutaneous reaction by the intravenous injection of endotoxin. HALPERN [8] succeeded in inhibiting this reaction with a polypeptide of antiprotease action. It is believed that in the local tissue damage associated with the Shwartzman reaction the tissue destructive enzymes released from the leukocytic lysosomes are of primary importance.

Concerning the pathogenesis of the local Shwartzman reaction, ANTOPOL and CHRYSSANTHOU [2, 3] wrote about the tissue response to endotoxin as

follows: "... the classical Shwartzman phenomenon involves several tissue responses mediated by multiple agents, including bradykinin and serotonin, which are sequentially or simultaneously released in a chain reaction initiated by endotoxin". SCHAYER [9] as well as HINSOW *et al.* [10] pointed to the central role of endotoxin in the histamine-releasing effect. Our findings appear to

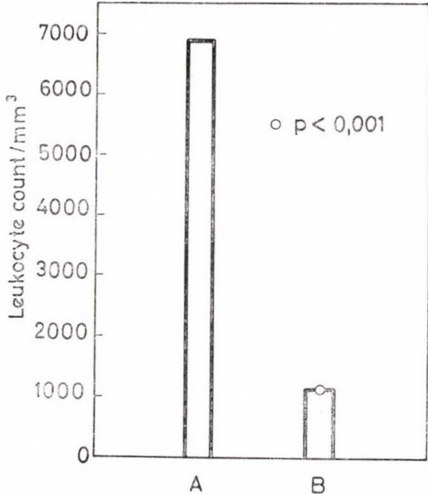

Fig. 1. Change of leukocyte count under the influence of dibromomannitol treatment. A: control; B: 48 hours after treatment with dibromomannitol

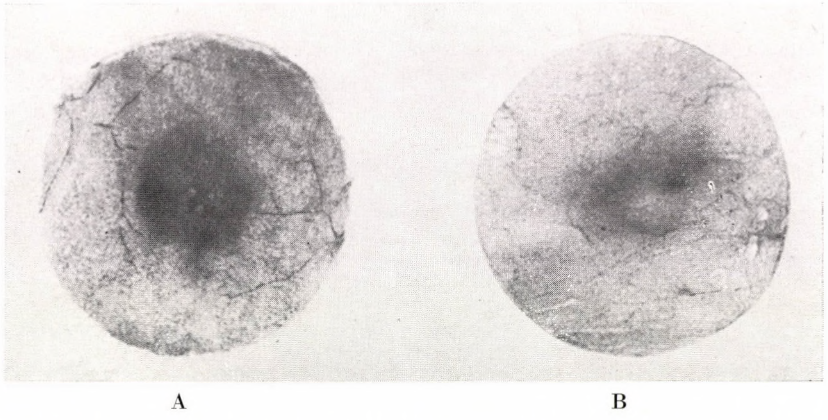

Fig. 2. Capillary permeability. A: control; B: pretreated with dibromomannitol

agree with the theory offered by BECKER [1] in that dibromomannitol, in addition to inducing leukopenia, seems to alter tissue reactivity. In rats pretreated with dibromomannitol there was a marked suppression of HALPERN—BRIOT's capillary permeability increase and of the anaphylactoid reaction as

well. Considering that in the dextran-induced anaphylactoid reaction of the rat the release of histamine and serotonin plays a decisive role [11] and these substances are of importance also in the pathogenesis of the local Shwartzman reaction, it seems likely that its suppression by nitrogen mustard and dibromomannitol is due not only to the induced leukopenia but also to the alteration of tissue reactivity.

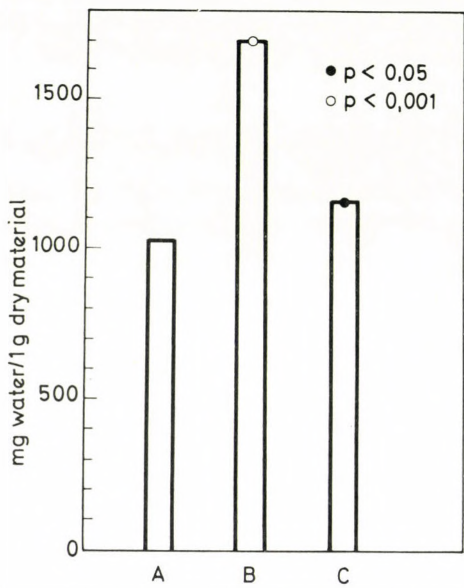

Fig. 3. Anaphylactoid reaction as assessed by the water content of the hind legs. A: control, untreated; B: control, after dextran treatment; C: treated with dibromomannitol and dextran

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BACTERIOLOGICAL MODEL EXPERIMENTS ON RADIOSTERILIZATION OF CATGUT

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Summary. The sterilizing dose of ionizing radiation has been determined by examining the natural bacterial contamination of gut samples and performing model experiments with sheep intestine intentionally contaminated with aerobic spore-bearing bacilli.

The D_{10} value was about twice higher for spores enclosed in the inside of catgut sutures than for spores dried on paper discs, *i.e.* a twofold dose was required for sterilizing catgut samples loaded with the same number of spores. On the basis of the findings, a dose of 3.5 *Mrad* ensuring a bacteriologically satisfactory safety factor (10^6) is recommended for catgut sterilization.

Radiation sterilization of surgical suturing material and other medical items has been used since 1958. Industrial-scale application of the method was first introduced in the U.S.A., then in England, Canada and Australia. Technical difficulties in the sterilization of catgut, a heat-sensitive product, have forced the industry to seek for methods ensuring more effective sterilization and less injurious processing than the customary chemical treatment. Introduction of radiation sterilization has been enhanced by the obvious advantage that this technique permits sterilization in the final, sealed container. In this manner the difficult and expensive packaging process is dispensed with and contamination of the material during packaging is perfectly prevented.

The first studies, based on practical observations, led to the choice of a 2.5 *Mrad* sterilizing dose for catgut [1, 2]. It is known that the number of bacteria highly influences the effective dose of radiation and several authors have pointed out that the empirical 2.5 *Mrad* dose is sufficient only when the level of contamination can be limited to a certain degree. The question is, accordingly, what is the lowest dose yielding an acceptable safety at a given level of contamination.

The problem may be approached in two manners. One of them is to subject preparations subjecting their natural contaminant flora to various doses of irradiation, then to perform sterility tests. The use of the customary sterility tests as an indicator of the effectiveness of sterilization has recently been criticized by some authors [3—5]. It has been concluded that the dose requirement and the margin of safety can more reliably be calculated by (1) determination of the contamination level before sterilization, (2) examina-

tion of the effectiveness of the given sterilization method in model experiments, (3) reliable physical control of the dose parameters of the sterilizing agent [6].

We have performed experiments according to the above, in certain minor details debated, but in basic features correct, principles. We examined sheep intestine, the raw material of catgut production, for natural bacterial contamination using samples exposed and not exposed to various processing techniques. For radiation sterilization experiments we elaborated a test model yielding data as to the protective action of animal intestinal tissue on the contaminating flora.

Materials and methods

Raw intestine and catgut preparations. Three different lots of dried raw sheep intestine shipped from two different plants were tested. *Non-iodized gut.* The intestine was treated with sodium hydroxide and potassium carbonate, then cleaned mechanically, twisted and dried. *Iodized catgut.* The gut strands were treated as indicated above, then exposed to dilute iodine solution. *Formalinized gut* was prepared in this manner except that formalin was substituted for iodine solution. The sutures were prepared from two strands of longitudinally halved guts; they corresponded in thickness to 0—00 according to the usual marking.

Viable counts. The samples were cut into pieces 10 cm in length, then homogenized by grinding in saline. Appropriate aliquots of the homogenate were plated onto blood agar plates. Other aliquots were mixed in Petri dishes into 10 ml portions of thioglycolate agar medium at 45 °C; after hardening the plates were overlayered with 20 ml thioglycolate agar medium. It was assumed that the thioglycolate medium would promote the growth of both aerobic and anaerobic bacteria and thus the difference between colony counts on blood agar and on thioglycolate agar would indicate the number of anaerobic organisms. Both kinds of medium were incubated at 37 °C for 48 hours.

Organisms used in model experiments. Bacillus pumilus E 601, *B. subtilis* subsp. niger NCTC 10073 and *B. stearothermophilus* NCIB 8919.

Preparation of spore suspensions. The organisms were cultured on sporulation agar [7] in Roux flasks at 37 °C (*B. pumilus* and *B. subtilis*) or 56 °C *(B. stearothermophilus)* until malachite green—safranine staining indicated at least 90% sporulation. The culture was then washed off with distilled water, passed through G2 Jena glass filter and washed three times in distilled water. The suspension was homogenized by shaking with glass beads, then filtered through G3 then G4 filters. Finally the spores were boiled for 5 minutes and, to promote the autolysis of vegetative cells, stored for 1 week at 4 °C.

Preparation of models. Viable counts in the basal suspensions prepared as described above were determined by preparing pour plates with glucose-Tripkazin (tryptic digest of casein) agar [7]. The basal suspensions were diluted to the required spore counts with distilled water. Paper discs 12 mm in diameter, prepared from Macherey-Nagel No. 214 paper, were impregnated so as to contain 10^6 spores per disc and dried at room temperature.

Sheep intestine was prepared as follows. Dry specimens of one lot were cut into strands about 1 metre in length, soaked, washed and pre-sterilized with a 2.5 *Mrad* dose of gamma radiation. The diluted spore suspension was sucked up into a tuberculin syringe fitted with a No. 1 Record needle. One end of the intestine was ligated with sterile suture; at the other end the needle was inserted into the lumen and the whole intestine was pulled up on the needle so that the tip of the needle reached the ligature. Then at 4 cm distance from the ligature (near to the socket of the needle) a thread was loosely tied around the intestine. After injecting 0.1 ml spore suspension (containing 10^6 spores) into the part separated in this manner, the needle was withdrawn beyond the second ligature and the knot was fastened. Thus, by ligating consecutive parts of the intestine and injecting spore suspension into the separated parts, the intestine was artificially contaminated in its full length. The wet specimens were gently twisted in by hand, then dried. Drying at room temperature was unsatisfactory for our purpose because it caused the spores to germinate. The following technique gave adequate results: the samples were placed in a vacuum chamber heated to 40 °C and the pressure was reduced to a few Hg mm. Thermistor measurements indicated that, in consequence of a high evaporation, the samples cooled rapidly to about 3 °C and kept this temperature throughout drying.

The dried intestines were placed in a chamber with 40 to 60% relative humidity for 24 hours, then cut into pieces at the site of the ligatures. In this manner "air dried" catgut samples each containing 10^6 spores of the tested organism were prepared. The artificially contaminated paper discs and catgut samples were placed between sterile filter paper strips and sealed separately in 0.05 mm thick polyethylene foil envelopes.

Radiation source. A ^{60}Co gamma emitter of 2.10^5 *rad*/hour capacity was used.

Recovery of the organisms. The irradiated test samples were cultured separately in broth containing glucose, Tripkazin, starch and bromcresol purple indicator at 37 °C (*B. pumilus* and *B. subtilis*) or at 56 °C (*B. stearothermophilus*) for at least 14 days. Bacteria cultured from the control and irradiated samples were checked for identity.

Analysis of the results. Direct inactivation curves were not determined since there was no way to obtain a complete recovery of survivors from the dry gut samples. Instead, an indirect method based on statistical probability was used for both paper discs and catgut preparations. Applying HALVORSON and ZIEGLER's [8] equation

$$\bar{x} = 2.3026 \log \frac{n}{q} \quad (1)$$

where $\bar{x}$ = the most probable number of spores surviving in one sample, n = the number of samples irradiated with the given dose and q = the number of samples found sterile on culturing; the total number of surviving spores was estimated by multiplying $\bar{x}$ with the number of samples irradiated with the same dose. For drawing the survivor curves the initial viable spore counts and — considering the shoulder in the inactivation curves at small doses — the value of the lag factor had to be known. The lag factor is the $\log_{10}$ surviving fraction expressed by the value above the 0 dose point (original number of organisms) where the tangent of the dose-survivor curve intersects the Y axis. The initial number of spores incorporated in the samples was known, the value of the lag factor was obtained from the literature [9].

The D_{10} value, in other words the dose needed for decreasing the number of viable spores by 90%, was estimated from the equation

$$D_{10} = \frac{D}{(\log a - \log b) + \text{lag factor}} \quad (2)$$

where a = the number of spores exposed to D dose (in our experiments the number of spores in one sample multiplied by the number of samples irradiated at the given dose); and b = the total number of spores surviving exposure to D dose (the number of spores surviving in one sample as estimated from Equ. (1) multiplied by the number of samples tested). The value of the lag factor had to be included in Equ. (2) because, due to the lag phase, the inactivation curve was not exponential throughout its whole course.

Results

The natural contamination of various gut samples and sutures treated in different manners is presented in Table I.

Table II shows the survival of spores on paper discs.

Paper discs and gut samples contaminated artificially with the same organism were sterilized in one cycle. From the control samples cultured parallel with the irradiated ones the organisms were always recovered. The results of experiments with intentionally contaminated gut specimens are shown in Table III.

If survival data for gut samples are compared with those for paper discs (Table II), it is evident that the radiation resistance of spores enclosed in the inside of the gut is significantly higher than of those dried on filter paper: the smallest dose needed to obtain sterility for all samples increased from 1.5 *Mrad*

Table I

Viable counts for natural contaminants in various raw gut strand and catgut suture samples

Preparation*	No. of samples	Average No. of bacteria			Highest No. of bacteria (aerobic + anaerobic)
		aerobic	anaerobic	total	
1. Raw sheep gut strands					
Lot A	10	4202	2 203	6 405	25 25C
Lot B	10	6653	19 193	25 846	45 500
Lot C	10	1571	454	2 025	9 650
2. Non-iodized catgut	6	.	.	19 144	28 400
3. Iodized catgut	6	.	.	18 689	30 800
4. Formalinized catgut	15	.	.	62	115

* Gut strands or sutures 10 cm in length.

Table II

*Survival of spores dried on paper discs**

	Per cent paper discs giving positive cultures after irradiation with						
	— (control)	0.5 *Mrad*	1.0 *Mrad*	1.5 *Mrad*	2.0 *Mrad*	2.5 *Mrad*	3.0 *Mrad*
B. pumilus E 601	100	100	90	—	—	—	—
B. subtilis subsp. niger NCTC 10073	100	100	—	—	—	—	—
B. stearothermophilus NCIB 8919	100	100	70	—	—	—	—

* Ten discs, each contaminated with 10^6 spores, were irradiated with each dose.

Table III

*Survival of spores dried in the inside of gut**

	Per cent of gut samples giving a positive culture after irradiation with							
	— (control)	0.5 *Mrad*	1.0 *Mrad*	1.5 *Mrad*	2.0 *Mrad*	2.5 *Mrad*	3.0 *Mrad*	3.5 *Mrad*
B. pumilus E 601	100	100	100	70	30	10	—	—
B. subtilis subsp. niger NCTC 10073	100	100	100	40	10	—	—	.
B. stearothermophilus NCIB 8919	100	100	100	60	10	—	—	.

* Ten gut samples, each contaminated with 10^6 spores, were irradiated at each dose.

to 3.0 *Mrad* for *B. pumilus* and from 1.5 *Mrad* to 2.5 *Mrad* for *B. stearothermophilus.*

Of the inactivation curves determined indirectly (Equ. 1), the curve for *B. pumilus*, an organism widely used in radiation resistance studies, is presented (Fig. 1).

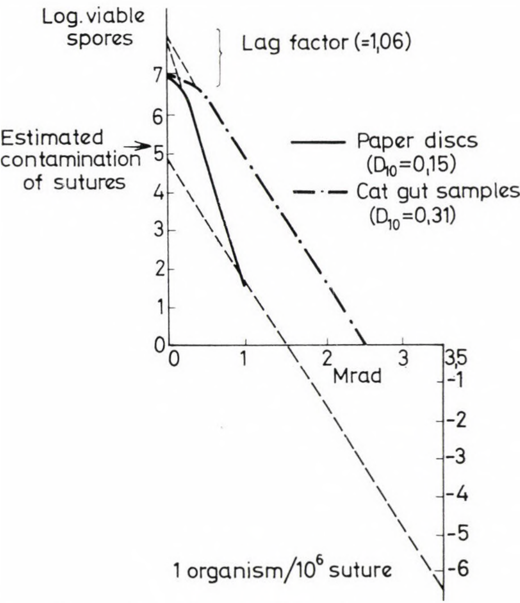

Fig. 1. Survival of *B. pumilus* spores and probability of contamination of catgut sutures sterilized with 3.5 *Mrad* doses

Accordingly, spores enclosed in catgut samples and dried on paper discs differ in radiation resistance. The difference can be expressed by D_{10} values determining the slopes of the two inactivation curves. The D_{10} values for *B. pumilus* estimated from Equ. (2) are: paper discs $D_{10} = 0.15$ *Mrad;* catgut $D_{10} = 0.31$ *Mrad.*

Discussion

Contaminating microorganisms in catgut sutures originate mainly from the intestinal flora of animals supplying the raw material. The quantitative and qualitative composition of the contaminating flora varies greatly with individual samples (see Table I). From Table I it is also evident that mechanical cleaning, washing and even mild iodine treatment exert practically no influence on the degree of bacterial contamination. Formalin treatment, however, reduces the germ count substantially.

Spores dried on paper discs were moderately resistant to radiation. This is only natural since the spores were washed several times in distilled water and the discs were impregnated with aqueous spore suspensions. Similar observations were made on *B. pumilus* by DARMADY *et al.* [10], CHRISTENSEN and HOLM [11] and LEY and TALLENTIRE [3]. There is no significant difference between our results and other authors' data [10, 12] in respect to the resistance of *B. subtilis* and *B. stearothermophilus*.

BURT and LEY [13] showed that the D_{10} value for *B. pumilus* E 601 was 0.17 under aerobic and 0.31 under anaerobic conditions. Our experiments with spores dried on paper discs and in the inside of gut gave similar results. We have concluded therefore that the increased radiation resistance of spores in catgut is due to anoxic conditions prevailing in the inside of the suture. As the D_{10} value for spores in catgut is about twice higher than that for spores on paper discs, it is evident that for destroying spore-bearing contaminants in sutures, the radiation dose sufficient for inactivating surface contaminants should be doubled.

Raw and pre-treated gut samples examined in this study were highly contaminated. In gut strands 10 cm in length the level of contamination was 10^4. From this finding it follows that each 2 metre portion of the thickest (No. 6) suture prepared from 4 sheep gut strands would contain 10^6 to 10^7 viable organisms before sterilization. Fig. 1 indicates that the inactivation factor for *B. pumilus* at the usual 2.5 *Mrad* dose is 10^7. It is clear that this dose fails to ensure an adequate safety margin for products showing such a high level of contamination. Pre-treatment with formalin is very effective: it decreases the expected level of contamination to 10^4 to 10^5 viable bacteria per strand. If formalinized catgut is irradiated with 2.5 *Mrad* doses, the safety factor corresponds to 10^3 to 10^2, that is, one survivor is present in every hundredth to thousandth suture. This level of survival cannot be considered safe. We propose accordingly that catgut sutures after processing with formalin should be exposed to 3.5 *Mrad* radiation. The inactivation factor ($10^{D:D_{10}}$) for *B. pumilus* in gut samples at the corresponding dose is 10^{11}. If the maximum contamination is 10^5 organisms per suture, and the contaminants are similar in resistance to *B. pumilus*, at most one viable spore will be present in 10^6 sutures. This value is acceptable. The same safety factor was recommended by RUBBO and GARDNER [14]. Our own views as to the 3.5 *Mrad* sterilizing dose coincide with those of MÉSZÁROS *et al.* [15] who recommended this dose on the basis of empirical findings. Finally it should be emphasized that the above safety margin is valid only if the contamination of the packaged catgut ready for irradiation is under the level of 10^5 organisms per suture. A higher level of contamination is unacceptable not only because it does not ensure an effective sterilization but also because of the danger of pyrogenic effect. The 2.5 *Mrad* dose applied by some firms may be suitable for sterilizing catgut preparations with lower

levels of contamination. Thus according to VAN WINKLE, BORICK and FOGARTY [9] the maximum pre-irradiation level of contamination in catgut sutures manufactured by Ethicon, Inc. was not more than 5.62×10^2 organisms per suture.

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STUDIES ON THE PERITONEAL EXUDATE OF ANIMALS EXPERIMENTALLY INFECTED WITH TOXOPLASMA GONDII*

I. EFFECT OF HEAT ON ACTIVITY

By

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Summary. The supernatant of the peritoneal exudate of Swiss white mice infected intraperitoneally with *T. gondii* causes convulsions and kills mice on intravenous injection. The same fluid from mice freshly succumbed to toxoplasmosis produces a similar effect. The toxic material was thermostable at 37 °C, 50 °C and was thermopotentiated at 56 °C for 30 minutes. Thermopotentiation at 56 °C was also obtained with diluted and with frozen-thawed toxic fluids. All materials treated at 56 °C regularly showed a visible increase of opalescence and an apparent increase of resistance to flow. Dilution with an equal volume of physiological saline, as well as freezing and thawing decreased but did not abolish its toxicity. Saline washings of the peritoneal exudate filtered through 0.7—1.5 μ glass filter became non-toxic. A tentative explanation of thermopotentiation and the nature of the active substance are discussed.

Several investigators [4, 5, 6, 8, 9, 10, 11, 12, 14, 16] have shown the presence of a toxic material in the supernatant of toxoplasmic peritoneal exudate of laboratory animals. The material is toxic only by the intravenous route. It has a spectacularly rapid action, inducing respiratory and nervous manifestations and in proper doses death in a few minutes. It is stable at room temperature, non-dialysable, non-filterable. It is a protein, mainly a globulin combined with hyaluronic acid.

There is, however, no agreement concerning the following points. (a) Behaviour of the toxin on heating at 37 °C, 50 °C, and 56 °C. (b) Its antigenicity. (c) The site and mechanism of action when injected intravenously in animals is not yet determined; colloid osmotic effect, viscosity (embolism), LSD diethylamide(D-lysergic acid) and circulatory disturbances have been made responsible for the manifestations and death. In this connection observations have been limited to symptomatology while the post mortem findings and histological studies are lacking. Similarly, the role of the toxic material in the toxoplasmosis syndrome is not yet clear. (d) Its origin whether from the mouse or the parasite, has not yet been settled.

The aims of the present study were (a) to test the effect of intravenous injection of mouse toxoplasmic peritoneal exudate into normal mice; (b) to test the effect of heat on the exudate's activity.

* Adapted from a Thesis submitted to the Hungarian Academy of Sciences.

Materials and methods

Toxoplasma strain. The RH strain of *T. gondii*, maintained in this laboratory by mouse passage twice weekly, was used in the experiments. The strain killed mice in 3—5 days.

Animals. Male and female white mice of a single parent Swiss strain were used. Unless otherwise specified, the animals weighed from 20 to 25 g. They were evenly divided among test and control groups in all experiments and all animals were maintained on a standard diet.

Methods. Mice were inoculated intraperitoneally with 0.3 ml of about 1/5 dilution in physiological saline of peritoneal exudate of mice infected intraperitoneally with *T. gondii* (TPE). On the fourth day these mice were killed by fracturing the neck, the abdominal skin was swabbed with alcohol and opened, and through the intact abdominal muscles TPE, mostly collecting in the flanks, was aseptically aspirated using a syringe and an 18 gauge needle. Individual lots were left to clot overnight at 5 °C. They were tested for sterility on blood agar. Sterile lots were pooled and centrifuged at 2000 r.p.m. for 30 minutes. The supernatant (SPET) thus was drawn and stored at 5 °C and used as toxin within a week.

Toxoplasma counts in freshly drawn TPE, thoroughly shaken with glass beads, were done using a Bürker chamber.

SPET was similarly obtained from mice freshly dead of toxoplasmosis.

Trials were made to concentrate the supernatant of centrifuged washes of TPE by a slight modification of KOHN's method [15] for the concentration of small amounts of protein solutions. The supernatant was put in diffusion tubes (No. 579 Schleicher, Schüll) and outside the tubes dextran powder (Kolidon 4%, Reanal, Budapest) was put. Attempts were also made to sterilize these washings by filtration through sintered glass filters (G5, Schott, Jena).

The effect of heat was studied as follows.

A. *Heating at 37 °C and 56 °C.* Pooled supernatant (PS) was divided into three parts, the first part was kept at 5 °C and served as control, the second part was incubated in a water bath at 37 °C for 30 minutes, and the third part was incubated in a water bath at 56 °C for 30 minutes (Table I).

B. *Effect of freezing and thawing and dilution on the potency of the exudate and its thermopotentiation.* PS was divided into two parts. The first part was diluted with an equal volume of physiological saline and then used in two fractions, one was assayed for the effect of dilution (Table II, exp. b) and the other incubated at 56 °C for 30 minutes and assayed for the effect of dilution on thermopotentiation (Table II, exp. c). The second part was used undiluted in three fractions, one served as control for the experiment (Table II, exp. a), the second was tested after subjecting it to rapid freezing in a dry ice—alcohol mixture and thawing ten times (Table II, exp. d) and the third after overnight deep freezing (—20 °C) and thawing twice (Table II, exp. e).

C. *Gradual heating to, and then maintenance at 37 °C, 50 °C and 56 °C.* PS was divided into four parts, the first part was kept at 5 °C and served as control, the second part was incubated in a water bath with the temperature gradually raised to 37 °C and then maintained for 30 minutes, and the third and fourth parts were similarly treated at 50 °C and 56 °C, respectively (Table III).

D. *Twice freezing (—20 °C) overnight and thawing followed by heating at 37 °C and 56 °C.* PS was divided into two parts, one was kept at 5 °C and served as control, the second was kept overnight in a deep freeze (—20 °C) and thawed twice, then divided into two parts; one part was incubated at 37 °C, and the other at 56 °C, for 30 minutes (Table IV).

E. *Rapid freezing and thawing ten times followed by heating at 37 °C and 56 °C.* PS was divided into two parts, one was kept at 5 °C and served as control, the second was rapidly frozen in a dry ice—alcohol mixture and thawed ten times, then divided into two parts; one was incubated in a water bath at 37 °C for 30 minutes, and the other at 56 °C, for 30 minutes (Table V).

Assay of activity was done by inoculation of PS into the tail vein of normal mice. PS kept at 5 °C, 37 °C and 50 °C was used undiluted. Fluid kept at 56 °C became opalescent, resisted flow in a narrow needle and that was why it was diluted with physiological saline before assay. Cold fluids were warmed and hot fluids cooled to 37 °C. Groups consisting of 5 mice each were given one of a series of gradually increasing doses of the exudate. Each dose differed by 0.001 ml/g from the preceding one.

The figures were calculated as dose/g; there was no need for a log dose transformation of the relative frequency calculated from the death rate belonging to a given dose in the different experiments and these values were the basis for calculation of the LD_{50} and the standard deviation (S.D.).

It was sometimes more convenient to reckon the difference in the death rate from the same dose of different PS (Table II).

Results

Character of mouse toxoplasmic peritoneal exudate (MTPE). The fluid was usually scanty until 48 hours before death, then it increased rapidly. It varied considerably in amount and physical character, being slightly turbid of various shades of yellow and was more or less viscous. The average amount harvested from one mouse was usually 0.5 to 1.0 ml; exceptionally amounts between 2 and 4 ml were obtained. The exudate contained Toxoplasma, leukocytes, proteins, and the toxic material. Toxoplasma counts after breaking down mouse cells by shaking the exudate with glass beads, varied between 200,000 and 400,000 organisms per cu.mm. After centrifugation the fluid became clearer.

SPET could be concentrated by dialysis against dextran. In a trial to filter the supernatant of saline washings of the peritoneal exudate through sintered glass filter under negative pressure, the process was slow and the filtrate was non-toxic. Some of the pools tended to form a tough mucoid precipitate when stored at 5 °C for 1—2 months and more markedly after heating at 56 °C and storing at —20 °C.

Toxic manifestations in mice following intravenous inoculation. A mouse receiving a lethal dose, usually 0.18—0.20 ml/20 g body weight, almost immediately rushed to and fro with the head and neck retracted backward and made several hops. Then it fell on its side or its back, kept motionless and apnoeic for a few seconds, then clonic convulsions of the limbs started, mainly kicking movements of the hind quarters, with passing of urine and sometimes faeces. Apnoea gave place to strong gasping movements, the mouth opening with each violent inspiratory effort, with gradually increasing exophthalmos, and then breathing ceased within a few minutes. Salivation was frequently present.

With smaller doses, the mouse moved its head up and down and sideways, sometimes it receded a little backwards and occasionally it rubbed its whiskers. Then it hurried in a waddling gait, or with its legs dragged behind ("froglike movement") or it leaned on one side, with the legs stretched on the opposite side and rotated on a vertical axis; sometimes it fell on its side, rolling around its own long axis (barrel rolling movement); or it kept still as it was laid on the table. For a few moments it was apnoeic and motionless, then suddenly righted itself up and apnoea gave place to hyperpnoea, which was sometimes periodic with 2 or 3 slow breaths followed by a number of rapid ones. With smaller doses there was usually no apnoea, the mouse showing a varying degree of weakness of the hind quarters and hyperpnoea.

Occasionally with medium and small doses some mice exhibited a primary phase of convulsions followed by an interval of 10 minutes to 1 hour during which the animal remained prostrated and hyperpnoeic, with livid snout, feet and tail. Then a second phase of convulsions occurred, a frothy sanguinous fluid flew from the nostrils and the animal died.

For a few moments after injection of the exudate the mice were hyper-excitable to auditory stimuli.

Sublethal doses of fluid diluted before heating at 56 °C caused fine tremors of body and tail after cessation of convulsions. Death usually occurred in 1—3 minutes, occasionally in 1 hour and rarely overnight.

Practically similar doses of PS aspirated immediately after death five days after infection, produced the same manifestations in normal mice and killed them as rapidly as did PS obtained from Toxoplasma-infected animals before death.

Effect of temperature on the potency of the exudate. Results are given in Tables I—V.

Table I shows that incubation of the exudate at 37 °C for 30 minutes did not affect its potency which was, however, increased to about three times after incubation at 56 °C for 30 min.

Table I

Effect of incubation at 37 °C and 56 °C for 30 minutes on the potency of the toxin

Group of mice	Material					
	Control kept at 5 °C		Incubated at 37 °C for 30 min		Incubated at 56 °C for 30 min	
	dose of toxin	No. of deaths	dose of toxin	No. of deaths	dose of toxin	No. of deaths
1	0.003	—	0.002	—	0.004	—
2	0.004	2	0.003	1	0.005	2
3	0.005	3	0.004	1	0.006	2
4	0.006	5	0.005	3	0.007	1
5			0.006	4	0.008	4
6			0.007	5	0.009	5
LD_{50}	0.0049		0.0047		0.0012	
S.D.	0.00032		0.00037		0.0009	
Confidence limits						
upper	0.0052		0.0054		0.0014	
lower	0.0038		0.0039		0.0011	

Table II shows that (a) dilution of the exudate with an equal volume of saline slightly affected its potency; (b) incubation of diluted fluid at 56 °C for 30 minutes increased its potency four times; and (c) freezing and thawing slightly decreased but did not abolish the potency.

Table III shows that the effect of 37 °C, 50 °C and 56 °C for 30 minutes was the same as described above, irrespective of whether the temperature had

Table II

Effect of dilution, dilution and heating, and freezing and thawing, on the potency of the toxin

Experiment No.	Material	Dose, ml/g body weight	No. of mice injected	No. of deaths	Per cent of deaths
II.a	Control	0.005	10	8	80
II.b	1 : 2 sln dln	0.010	10	6	60
II.c	1 : 2 dln and htg 56 °C	0.0025	10	6	60
II.d	10 times CO_2 FT	0.005	10	4	40
II.e	2 times DFT	0.005	10	4	40

sln = saline, dln = dilution, htg = heating
FT = freezing and thawing, DFT = deep freezing and thawing

Table III

Effect of incubation at temperatures gradually raised to and then maintained at 37 °C, 50 °C and 56 °C, on the potency of the toxin

Group of mice	Material							
	Control kept at 5 °C		Incubated at 37 °C for 30 min		Incubated at 50 °C for 30 min		Incubated at 56 °C for 30 min	
	dose of toxin	No. of deaths	dose of toxin	No. of deaths	dose of toxin	No. of deaths	dose of toxin	No. of deaths
1	0.004	—	0.004	—	0.004	—	0.004	—
2	0.005	2	0.005	2	0.005	2	0.005	2
3	0.006	3	0.006	3	0.006	4	0.006	4
4	0.007	4	0.007	4	0.007	5	0.007	5
5	0.008	5	0.008	5				
LD_{50}	0.0057		0.0057		0.0053		0.0011	
S.D.	0.0046		0.0036		0.00027		0.00005	
Confidence limits								
upper	0.0064		0.0064		0.0058		0.0012	
lower	0.0049		0.0049		0.0047		0.00095	

been raised gradually to and then maintained at, or adjusted to these temperatures.

Table IV shows that incubation at 37 °C for 30 minutes of twice frozen (—20 °C) and thawed exudate did not affect its potency which was, however, increased to six times after incubation at 56 °C for the same time.

Table V shows that incubation at 37 °C for 30 minutes of ten times rapidly frozen (dry ice—alcohol mixture) and thawed exudate did not affect its potency which was, however, increased to about four times after incubation at 56 °C for the same time.

Table IV

Effect of incubation at 37 °C and 56 °C for 30 minutes of twice frozen (—20 °C) and thawed toxin

Group of mice	Material					
	Control kept at 5 °C		Incubated at 37 °C for 30 min		Incubated at 56 °C for 30 min	
	dose of toxin	No. of deaths	dose of toxin	No. of deaths	dose of toxin	No. of deaths
1	0.004	—	0.004	—	0.003	—
2	0.005	1	0.005	1	0.004	2
3	0.006	3	0.006	2	0.005	4
4	0.007	5	0.007	3	0.006	5
5			0.008	5		
LD_{50}	0.0057		0.0063		0.00086	
S.D.	0.00026		0.00036		0.00005	
Confidence limits						
upper	0.0062		0.0071		0.00097	
lower	0.0052		0.0056		0.00075	

Table V

Effect of incubation at 37 °C and 56 °C of ten times frozen (CO_2—ice—alcohol mixture) and thawed toxin

Group of mice	Material					
	Control kept at 5 °C		Incubated at 37 °C for 30 min		Incubated at 56 °C for 30 min	
	dose of toxin	No. of deaths	dose of toxin	No. of deaths	dose of toxin	No. of deaths
1	0.004	—	0.004	—	0.006	—
2	0.005	1	0.005	1	0.007	1
3	0.006	2	0.006	2	0.008	2
4	0.007	4	0.007	3	0.009	4
5	0.008	5	0.008	3	0.010	5
6			0.009	5		
LD_{50}	0.0061		0.0068		0.0016	
S.D.	0.00032		0.00045		0.00006	
Confidence limits						
upper	0.0067		0.0077		0.0018	
lower	0.0054		0.0058		0.0015	

Discussion

The findings have confirmed the presence in SPET of a substance which on intravenous injection in mice gives rise to nervous and respiratory manifestations and to death. The exudate has a characteristically rapid action, taking a few seconds to produce symptoms and several seconds to a few minutes to produce death. SPET collected from mice immediately after they had succumbed to toxoplasmosis similarly kills normal mice, a finding which is in contrast with the data reported by Varela *et al.* [9] in that the peritoneal exudate of mice which succumbed to intraperitoneal infection with *T. gondii* failed to kill normal mice. This, however, does not prove or disprove the role of the exudate in the pathogenesis of toxoplasmosis.

The symptoms arising after the intravenous injection of SPET carried a resemblance to anaphylactic shock as described in the mouse [13] but required no sensitization.

The active material in SPET is non-dialysable [5, 6, 12, 16]. In this study the peritoneal washings were concentrated by dialysis against dextran. Filtration through 0.7—1.5 μ pore size filters if the supernatant of saline washings of the peritoneal exudate was slow and the filtrate was not toxic. This agrees with the findings of other authors. Thus Debatin [4] reported difficulty to filter SPET due to its viscosity, and Fulton [5] found a reduction of toxicity by filtration through filter paper and loss of toxicity after passing through a collodion membrane or Seitz filter. Motomura [16] reported a reduction of toxicity by filtration through filters of 0.45 μ pore size.

The active material in SPET was stable at 37 °C and 56 °C which agrees with reports by other workers [11, 12]. Gradual heating at 37 °C and 50 °C for 30 minutes neither increased nor decreased its potency and this does not agree with the finding of Machado *et al.* [6] who reported loss of activity by similar treatments.

Opinions differ as to the effect of heating at 56 °C for 30 minutes. One author observed inactivation [4], another author reported on stability [12]. Our findings, however, agree with those who reported potentiation of the toxicity at 56 °C [5, 11, 12, 14, 16]. Thermopotentiation was not inhibited by preceding freezing and thawing nor by preceding dilution.

It was reported that a lethal dose of cotton rat SPET diluted with an equal volume of saline no longer caused death [5]. In the present experiments, however, a similarly treated SPET from white mice remained toxic provided that a double dose was given from the diluted fluid.

Freezing and thawing do not cause a loss of toxicity [4, 5]. In the present study, toxicity was reduced but not abolished by freezing and thawing.

There is no sound basis to attribute the activity of SPET to Toxoplasma, ignoring the role of the greater part of SPET, namely the mouse proteins.

According to PETTERSEN [8], the toxic material appears to be of mouse rather than of parasitic origin. Using ammonium sulphate fractionation, activity was recovered with the globulin fraction of the mouse SPET [14]. The toxic material acts only on intravenous injection and the symptoms produced are similar to those of anaphylactic shock in mice [13]. As quoted by McKINLEY [7], BORDET found that the intravenous injection of fresh normal guinea pig serum globulin into normal guinea pigs is followed by shock and death of the animal in 3 or 4 minutes, the heart and lungs presenting a picture resembling that seen in anaphylactic shock. Intraperitoneal or subcutaneous injection had no effect.

Heating of SPET is accompanied by an increase in its opalescence, in viscosity, and in potency. The true cause of thermopotentiation is not exactly known. It may be the result of inactivation of complement, which is known to be destroyed by such a treatment. Moreover, heating at 56 °C causes denaturation of proteins and this is associated with the exposure of reactive groups that had been buried inside the protein molecule which consequently becomes more reactive with other molecules. Rapidly sedimenting components are formed and the 23S fraction increases [5]. Gamma-globulins heated to 56 °C show molecular aggregation and anticomplementary properties and cause anaphylaxis-like symptoms in some guinea pigs [2, 3].

The above mentioned data may explain the role of mouse globulins in the toxic activity of SPET and the nature of its thermopotentiation.

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REPLICATION OF VACCINIA VIRUS IN THE EMBRYONATED EGG IN THE PRESENCE OF METHISAZONE

AN ELECTRON-MICROSCOPIC STUDY

By

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Summary. Vaccinia virus was propagated in the chorionic cells of the chick embryo chorioallantoic membrane in the presence of 20 μM of methisazone per egg. Virus multiplication was followed by infectivity titration and electron microscopy.

(i) The early events until the eclipse phase were not different from the control.

(ii) In the first 24 hours of the cycle no young replicating forms were seen. The matrices which had appeared by the 48th hour were small in number, often indistinct in shape and irregular in structure. Groups consisting of a few immature virus-like bodies irregular in shape were seen in the matrix. The outer membrane of many particles was defective. The viroplasm of some of the immature forms appeared structureless. Mature and immature forms did not occur together in the same section. Mature virions, if present, were few in number.

(iii) Two hitherto undescribed phenomena have been observed. (a) Twenty-four hours after methisazone-treatment and subsequent infection the canaliculi of the granular endoplasmic reticulum were dilated and unusually increased in number in many of the cells. (b) Most of the mature virions were surrounded by a vacuole and two double-layered membranes.

In a previous report [1] the morphology of the vaccinia virus reproduction in the cells of the chorionic membrane of the embryonated egg was described. It has been concluded that for such morphological studies the embryonated egg is as suitable as are the usual cell cultures.

Furthermore, the effect of methisazone on the multiplication of vaccinia virus was studied in embryonated eggs, cell cultures and in the mouse brain [2, 3]. In the mouse-brain experiments a neurovirulent strain was compared with a non-neurovirulent one. In the presence of methisazone the rate of virus multiplication was significantly reduced.

Other investigators [4, 5, 6] found that some morphological characteristics of the virus replication are considerably altered by methisazone.

The present report describes the morphological characteristics of the vaccinia virus replication proceeding in the chorionic cells of the embryonated egg in the presence of methisazone.

Materials and methods

The virus strain, the preparation technique for electron microscopy and the virus titration have been described [1].

Inhibition of virus multiplication by methisazone. A 20 μM dose of methisazone was injected in a volume of 0.2 ml into the yolk of each of the eggs pre-incubated for 11 days. An arti-

ficial air sac was prepared and the chorionic side of the chorioallantoic membrane (CAM) was infected with 600 to 1000 egg-PFU of virus in a volume of 0.2 ml. For electron microscopy only those eggs were used in which the embryo was still living and moved normally. Further methodical details have been published elsewhere [2].

Results

Virus replication in the CAM. The curves in Fig. 1 illustrate the course of virus multiplication in CAM infected with 600 to 1000 egg-PFU of vaccinia virus. One group of eggs was injected with 0.2 ml of saline, the other with

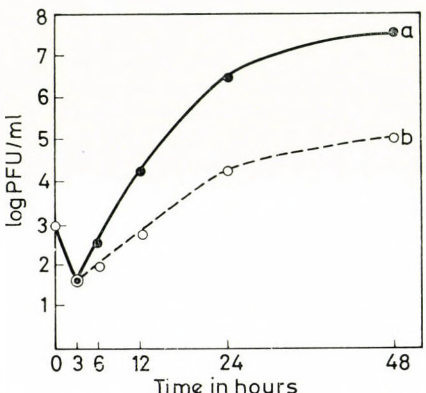

Fig. 1. Multiplication of vaccinia virus in the chick embryo CAM. (a) Control group; (b) embryos injected with 20 μM of methisazone per egg

20 μM of methisazone in the same volume of saline per egg. The control curve showed that logarithmic multiplication started 3 to 6 hours after inoculation. By the same time some multiplication had occurred in the methisazone-treated membranes as well, but by 12 hours the amount of the newly-produced infective virus had not reached that injected in the inoculum; at 24 and 48 hours the virus yield was less than 0.5% of the control.

Morphology of virus replication

Early events. The morphology of adsorption, penetration and turning into eclipse developed in both groups in the same manner as described earlier, *i.e.*, virions were adsorbed to cells and then engulfed like foreign bodies. In the foreign-body inclusions the virions soon lost their original structure. Subsequently, as a result of the enzymatic activity of the cell, the virions' envelope was decomposed and the DNA-containing viral core was released. Engulfed virions are shown in Figs 2 and 3. The virions lying free in the cytoplasm cannot be distinguished from extracellular virions. Arrows point to three inclusions surrounded by unit membranes. The double arrow points to a similarly enclosed

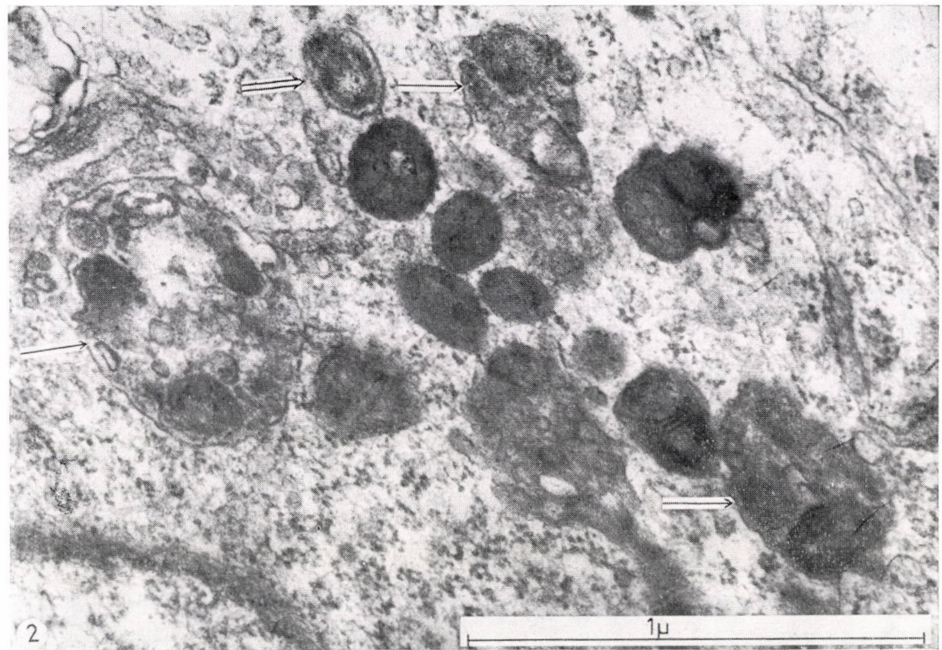

Fig. 2. (Photo No. 9214). Chorionic cell in methisazone-treated CAM 24 hours after inoculation with vaccinia virus. Single arrows point to two foreign-body inclusions containing virions turning into the eclipse phase. Double arrow points to a single virion in an inclusion. Magnification, ×61,000

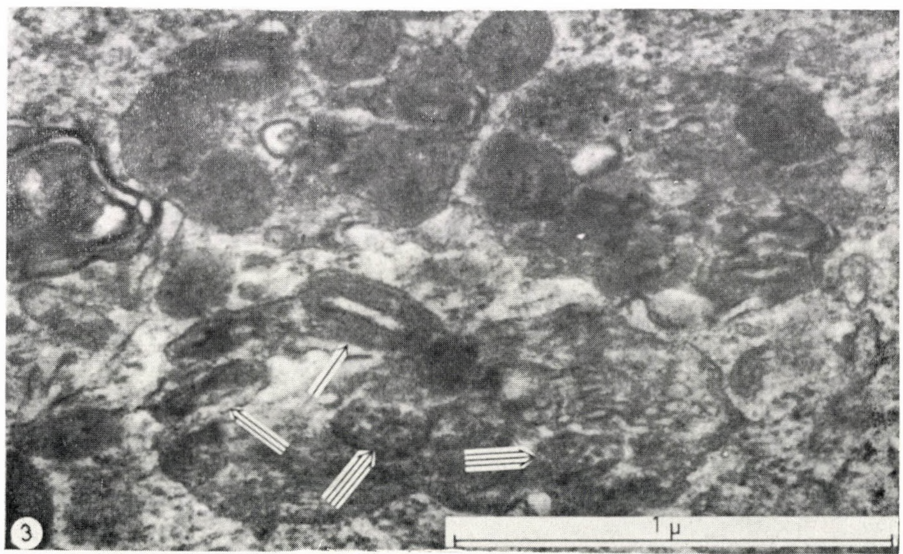

Fig. 3. (Photo No. 9203). Three foreign-body inclusions. Single, double and triple arrows poin to particles in different stages of turning into eclipse. ×58,000

single virion. The structure of these virions is injured, their envelope is indistinct. In the inclusions and in the neighbouring cytoplasm there are vesicles of variable size. Most of the inclusions and vesicles are limited by agranular membranes. The early disintegration of the virions on the left in Fig. 2, shown under higher power in Fig. 4, is more pronounced. In Fig. 3 three virus-containing inclusions and two free virions are seen. The latter are intact while the particles — six in the upper left inclusion, four in an inclusion on the right and four or more in the elongated one on the bottom — show various stages of disintegration of the outer components and liberation of the viral core. The single arrow points to a particle whose side adjacent to the wall of the inclusion appears intact, whereas the one at the point of the arrow is injured. The double outer membrane of the particle at the point of the double arrow is lacking. In the two formations pointed at by the triple arrow there are elements resembling the strands of nucleic acid released from the viral core. Their identification needs histochemical investigation. The viral bodies in the upper inclusion are variably indistinct. In Fig. 5 the lower elongated inclusion of Fig. 3 is presented at higher magnification.

Cytological lesions in the first 24 hours. In the not pretreated eggs the chorionic cells of the CAM showed an accumulation of mitochondria and appearance of filamentous or structureless inclusions 12 hours after infection (Figs 2—5 in [1]). Soon thereafter young forms appeared.

In the presence of methisazone no newly formed particles could be demonstrated in the first 24 hours. However, in such membranes a cell-form very rare in normal and vaccinia virus-infected CAM was frequently seen. These cells characterized by a striking abundance of granular canaliculi of the endoplasmic reticulum may be immigrant cells or altered chorionic cells.

In chorionic cells inclusions corresponding to Guarnieri bodies are rarely seen by light microscopy and, to our best knowledge, electron-microscopic inclusions like those common in vaccinia virus-infected rabbit cornea cells have never been observed in CAM cells.

The electron micrograph presented in Fig. 6 was taken 24 hours after the infection of a methisazone-treated egg. The nuclear structure is unaltered. In the cytoplasm the dilated canaliculi of the granular endoplasmic reticulum are increased in number. Besides, elements of normal, undilated canaliculi are present. Although the cell in the picture is rich in mitochondria, this is not characteristic of the methisazone-treated and infected cells. In Fig. 7, inside two inclusions, indistinct, moderately electron-dense spots variable in size and shape, and dense granules resembling ribosomes are visible. Near the right lower corner a small part of the dilated canaliculi of the endoplasmic reticulum appears.

Changes observed 48 hours after infection. Newly-formed immature virus particles, apparently absent in the first 24 hours, are visible at 48 hours. How-

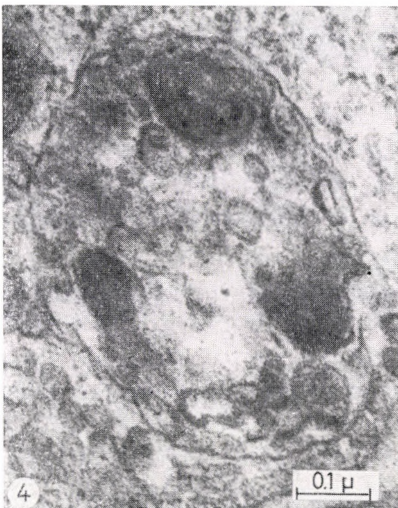

Fig. 4. Photo No. 9214; magnification ×92,000

ever, these are altered by the methisazone treatment. The nucleus under low-power (Fig. 8) shows a normal structure, but in the cytoplasm an increased number of vacuoles and a slight accumulation of endoplasmic reticulum are seen; part of the canaliculi is not dilated. The few immature forms are scattered in the cytoplasm, in contrast to those in the uninhibited chorionic cells,

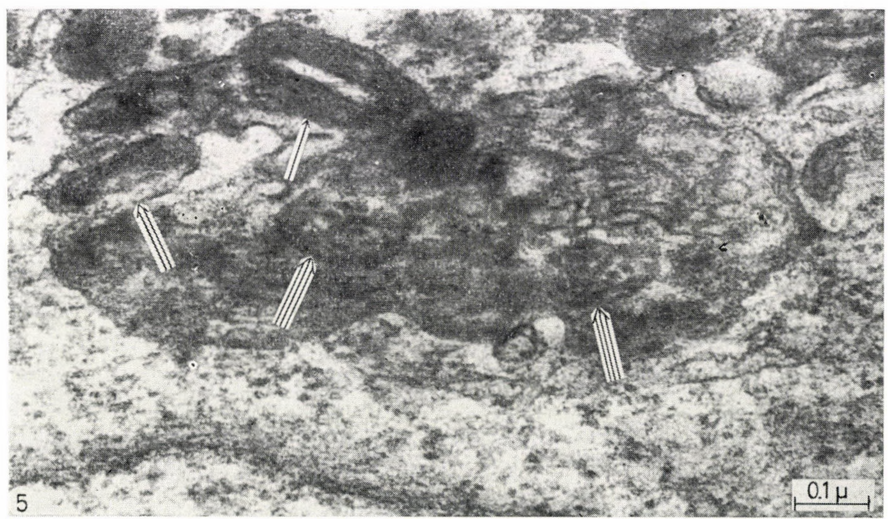

Fig. 5. The lower inclusion in Photo No. 9203; magnification ×88,000

in which the immature forms are located juxta-nuclearly. In some cells, e.g. in that shown in Fig. 9, a large group of immature particles is seen, and the matrix is loose, a phenomenon characteristic of an inhibited virus reproduction. Most of the immature particles are not oval in shape, some of them have excess limiting membranes (upper right corner), the membranes of others (on the right, near the midline) are defective. Occasionally, the viroplasm-matrix limit is hardly visible. The viroplasm is mostly of normal structure though particles containing a structureless, dense substance also occur. One of the immature particles has a young nucleoid, but no limiting membrane.

In Fig. 10 five immature particles are embedded in a matrix of normal structure. The particles are irregular in shape. In the micrograph only traces of the filamentous structure are detectable in the nucleoid. The matrix is surrounded by an abundant endoplasmic reticulum and ribosomes. In the cytoplasm there are loose filaments of unknown function.

The picture in Fig. 11 resembles that in Fig. 9. The structure of the matrix is indistinct. The limiting membranes of the immature particles are irregular, some even defective. Particles with partly structureless viroplasm also occur. The matrix is surrounded by canaliculi of the endoplasmic reticulum.

Mature virions. Among the 48-hour sections few contained mature virions. Thus, a complete series of developmental stages could never be seen in one and the same cell. While under normal conditions many of the virions are surrounded by endoplasmic reticulum, most of those developing in the methisazone-treated cells were surrounded by an electron-dense membrane duplicate or, occasionally, a vacuole.

In Fig. 12, on the left near the bottom, there are several mature virions lying free in the cytoplasm near the nucleus. The virion on the left is surrounded by membrane duplicates, probably agranular duplicates of the endoplasmic reticulum. These are more electron-dense than the endoplasmic reticulum elsewhere in the picture. There is often a vacuole between the double-layered membrane and the virion. Near the centre of Fig. 12 there is an empty vacuole. Foreign substance was rarely found in such vacuoles. In control preparations virions surrounded by vacuoles did not occur.

Fig. 13 shows a virion in a cytoplasmic process, surrounded by a small vacuole and two electron-dense double-layered membranes. Inside the vacuole the particle has limiting membranes characteristic of the mature virion. Inside the nucleoid a filamentous substance is visible.

In Fig. 14 a virion is just being discharged from a cytoplasmic process of a chorionic cell. The virion is separated from the cytoplasm by two double-layered membranes similar to those seen in the above Figures, and from the membrane it is separated by a vacuole. Towards the intracellular space the membranes are loose and partly disintegrated.

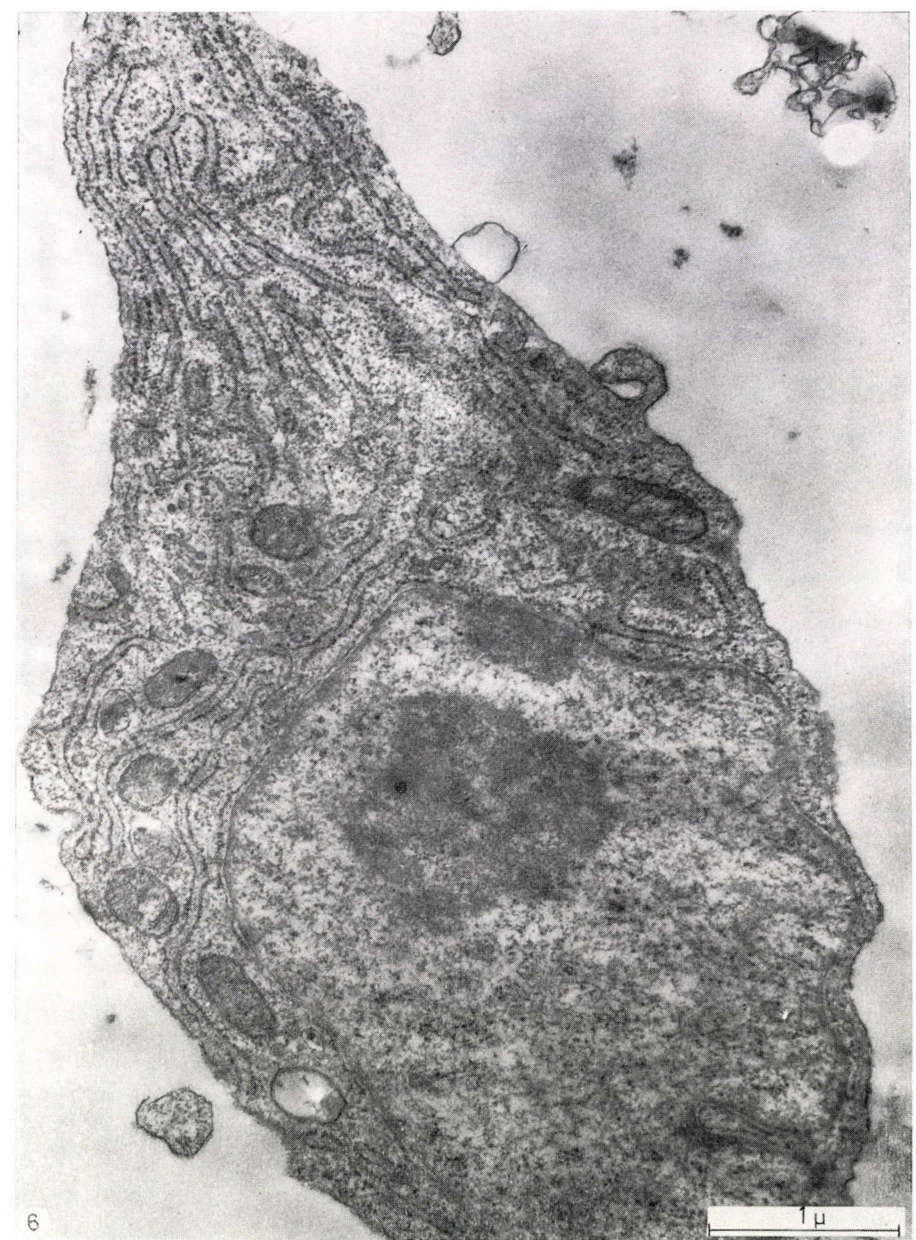

Fig. 6. (Photo 9162). Chorionic cells 24 hours after methisazone-treatment and infection. Abundance in endoplasmic reticulum, dilated canaliculi. ×24,000

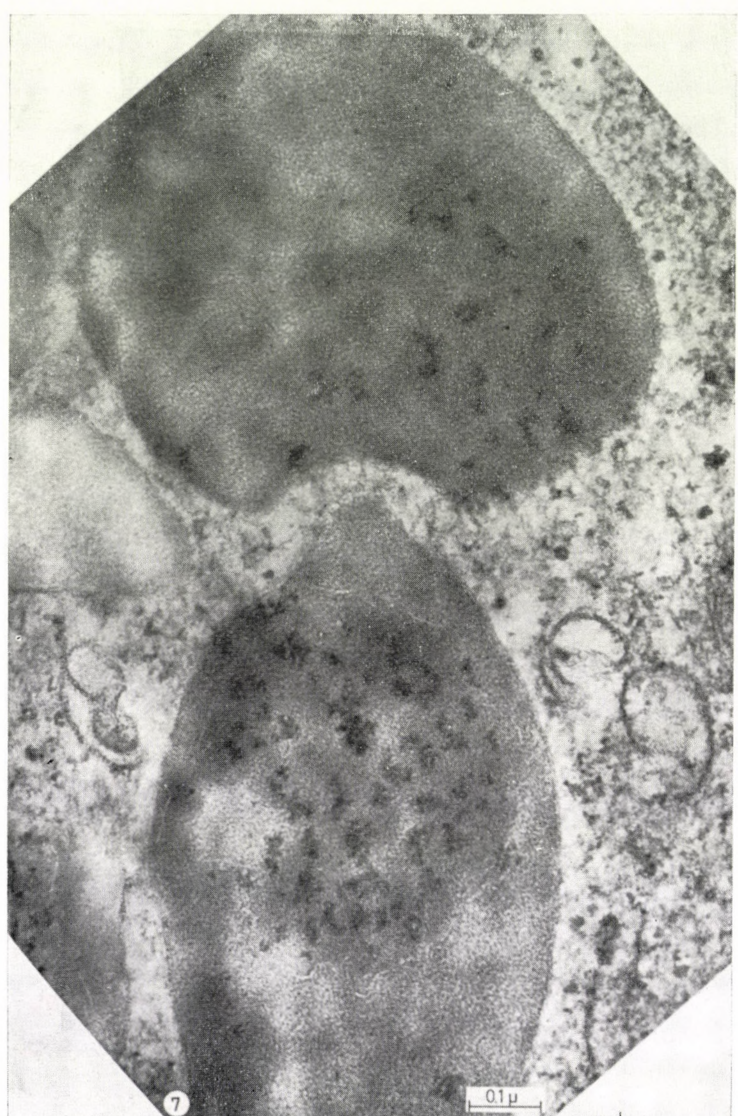

Fig. 7. (Photo No. 9160). Two juxtanuclear inclusions. ×112,000

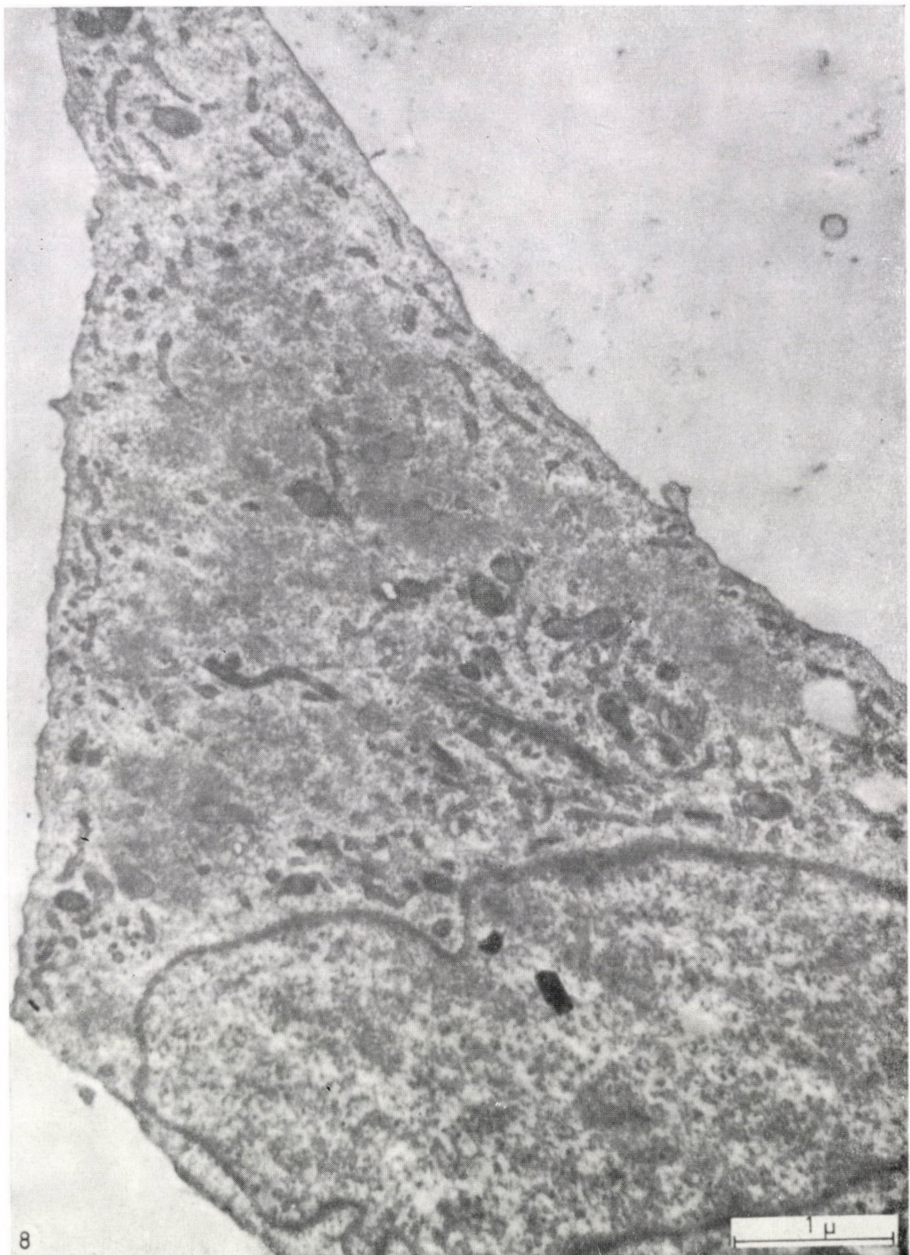

Fig. 8. (Photo No. 9163). Scattered immature virus-like particles in the cytoplasm 48 **hours** after methisazone-treatment and infection. $\times$ 20,400

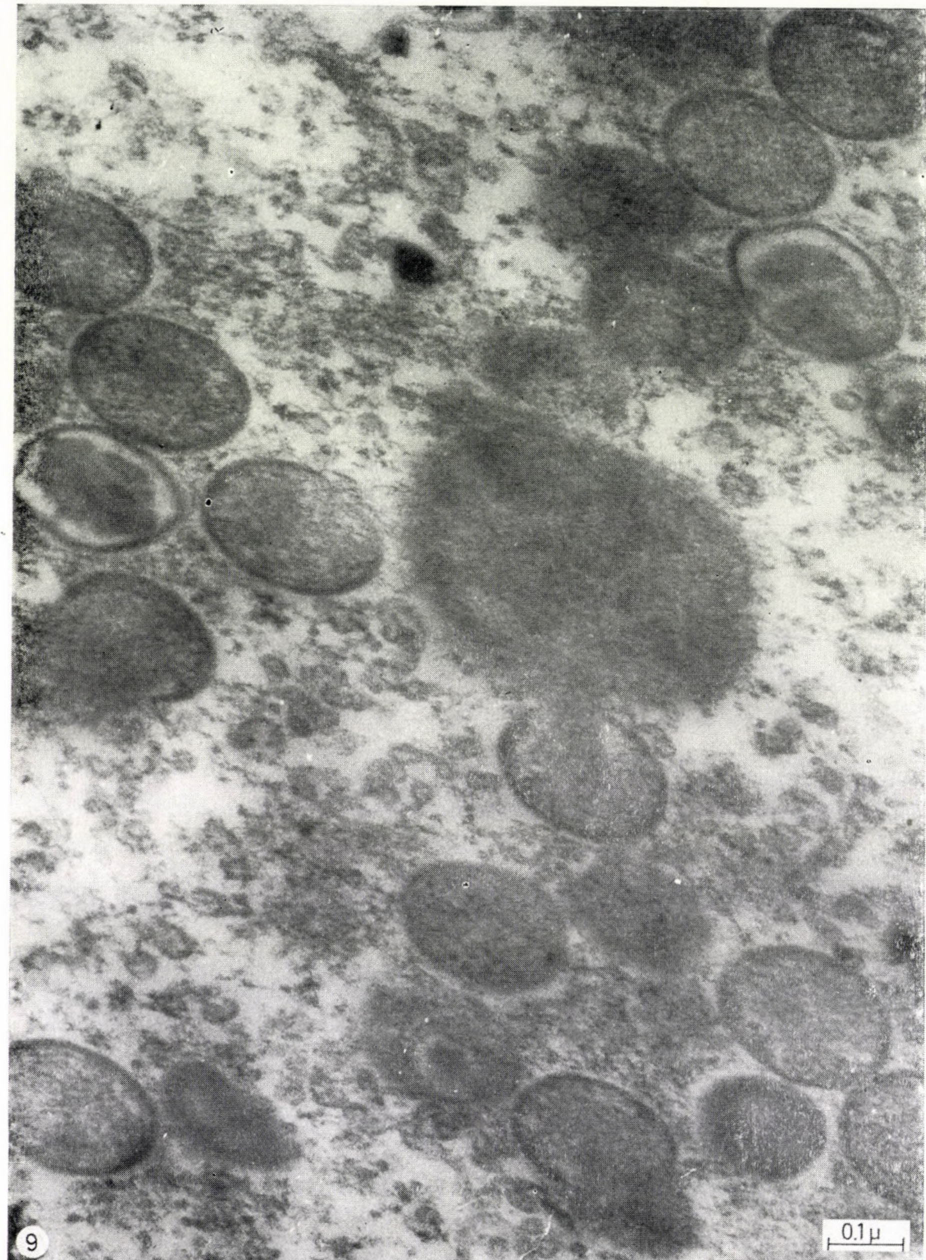

Fig. 9. (Photo No. 9174). Immature forms 48 hours after methisazone-treatment and infection. Magnification, ×90,000

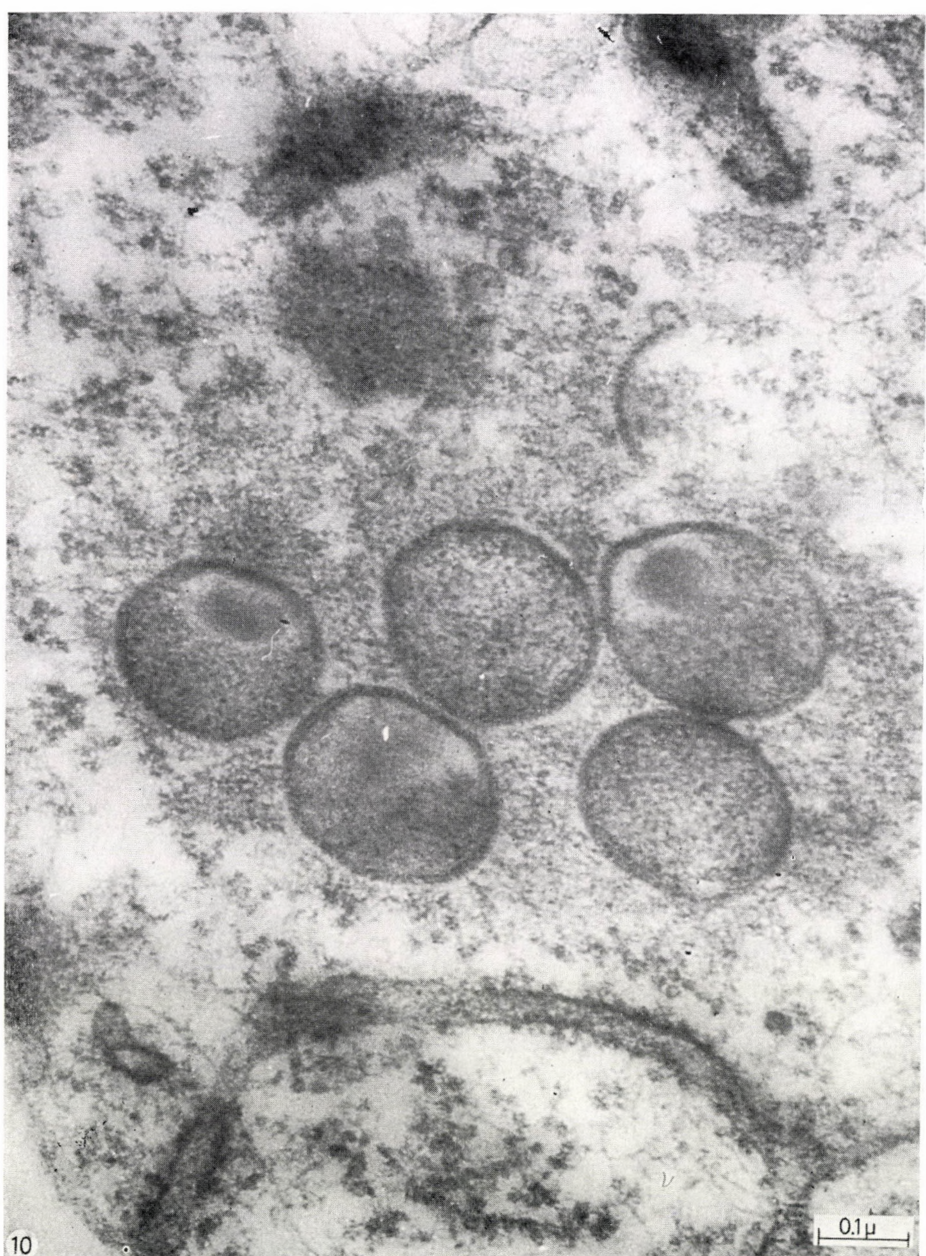

Fig. 10. (Photo 9174). Pattern similar to that in Fig. 8. $\times 120{,}000$

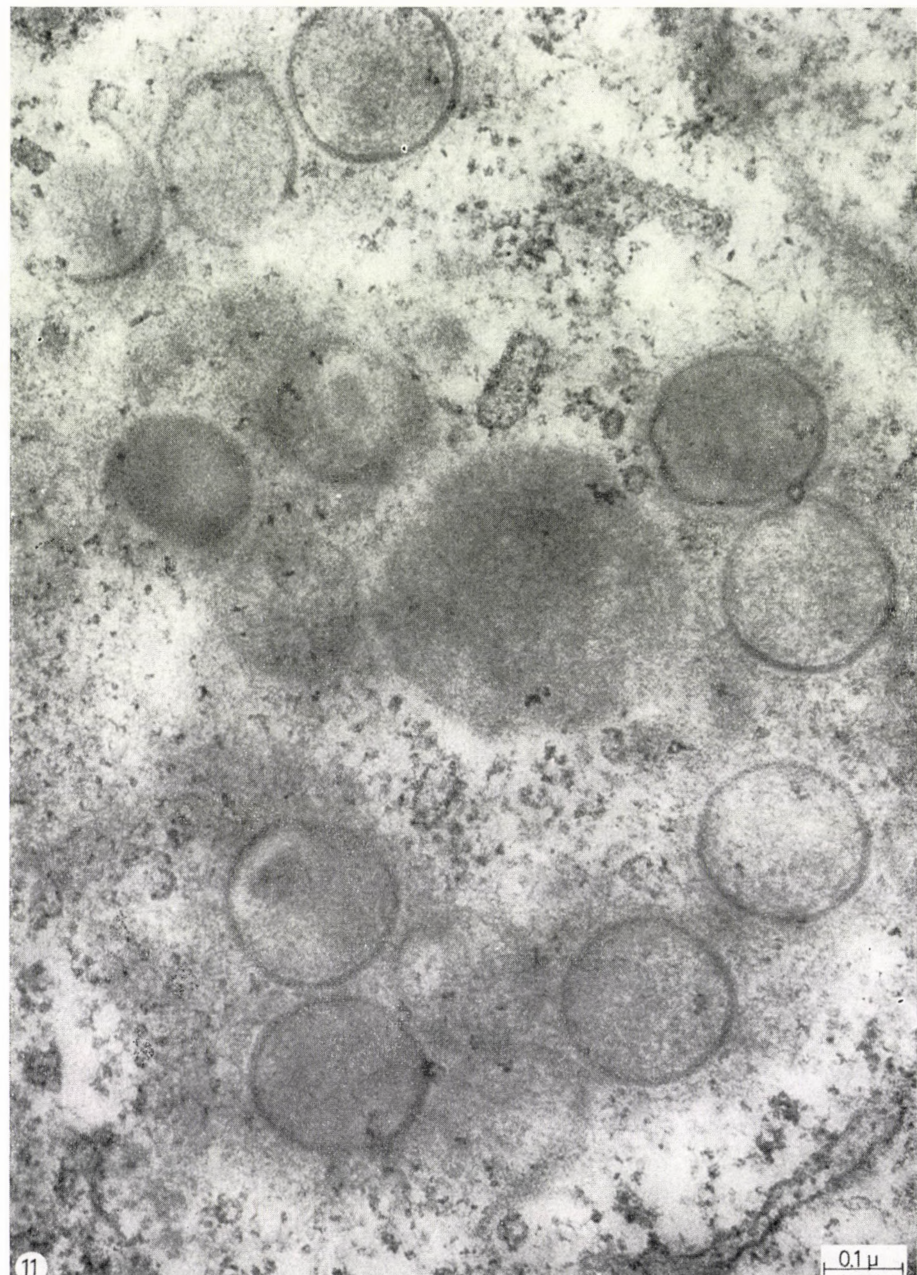

Fig. 11. (Photo No. 9144). Pattern similar as in Fig. 8. $\times$ 90,000

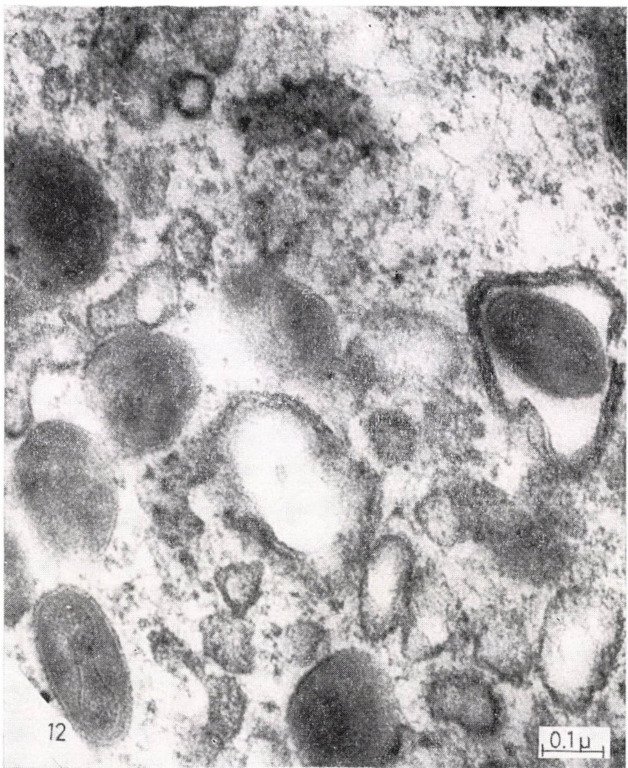

Fig. 12. (Photo No. 9164). Mature virions in cytoplasm, enclosed in two double-layered membranes of high electron-density. Note vacuole around each virion. ×80,000

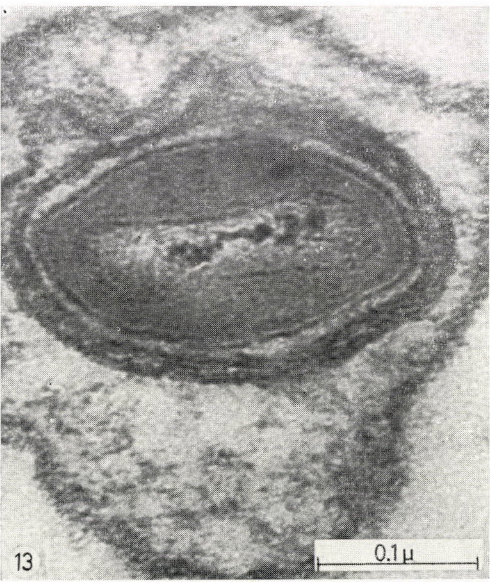

Fig. 13. (Photo No. 9181). In a cytoplasmic process a mature virion surrounded by a vacuole and two double-layered membranes. Note filamentous structure of nucleoid. ×214,000

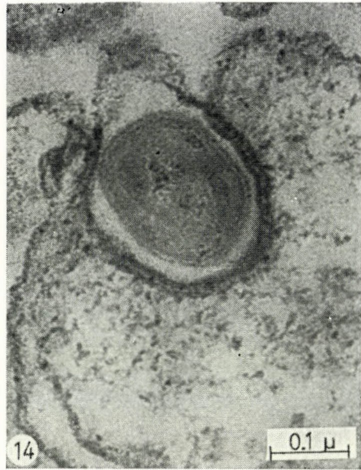

Fig. 14. (Photo No. 9142). Discharge of a virion at the cell surface. ×100,000.

Discussion

The literature on vaccinia virus replication has been reviewed previously [1]. Most of these studies as also those of the mechanism of action of methisazone and of morphological characteristics of the inhibited replication cycle were performed in tissue cultures.

In such studies we give preference to the embryonated egg since this allows a better comparison of the studied processes with those occurring in the living organism. This view is supported by the therapeutic index of methisazone in different virus-host systems [3, 7]. For the chick embryo fibroblast system this index was around 1.0, for the embryonated egg it ranged between 2 and 4, whereas for the mouse brain, depending on the virulence of the virus strain, between 10 and 32.

It would not have been surprising to find host-dependent morphological differences in the inhibition of replication. However, in the present study the course of the inhibited cycle was morphologically similar to both the normal cycle in the embryonated egg and the cycle proceeding in tissue culture in the presence of methisazone. In the chorionic cells of the embryonated egg injected with 20 μM of methisazone immediately before virus infection on the 11th day of incubation, the adsorption, penetration and turning into eclipse were indistinguishable from the respective events observed by us in the control CAM system or by others in tissue cultures. There were only quantitative differences in respect of the virus-producing matrix and the immature particles per factory. In view of the well-defined quantitative differences the reproduction cycle seems to be considerably inhibited as early as at the formation of

immature particles. Mature virions were few, and immature particles and mature virions were never found in the same section.

In spite of the morphological similarity, two hitherto undescribed phenomena have been observed.

(i) In the presence of methisazone a special type of cell was found in the CAM of vaccinia virus-infected embryos. These cells, which we believe to represent altered chorionic cells or immigrated histiocytes, possessed an increased amount of granular endoplasmic reticulum and dilated canaliculi. The fact that most of the apparently virus-producing cells were of this character, speaks for their chorionic origin. On the other hand, for their histiocytic origin speaks that such cells occurred even in CAM which had not been subjected to pretreatment and that the number of such cells increased after any manipulation, for instance on inoculation of saline onto the CAM.

(ii) Most of the mature virions were enclosed by two double-layered membranes. The membrane walls were agranular, yet, more electron-dense than the canaliculi of the endoplasmic reticulum. Such formations were seen only in the chorionic cells of methisazone-pretreated infected CAM.

The abundance in dilated canaliculi of the endoplasmic reticulum may be considered a morphological phenomenon accompanying a particular cell function, e.g., an enhanced production. This view is supported by literary data concerning the mechanism of action of methisazone. According to APPLEYARD *et al.* [8], in methisazone-treated cells the vaccinia virus induces the synthesis of a new protein which, blocking certain polyribosome—viral RNA complexes, disturbs the synthesis of an enzyme or structure protein indispensable for virus maturation. The cytomorphological phenomena described in the present report might be explained by an enhanced synthesis of the blocking protein.

Alternately, the cytomorphological phenomena might be attributed to the toxic effect of methisazone. In this case, however, a similar phenomenon should have been observed in chicken fibroblast cultures, as in this system the drug has a low therapeutic index.

The high incidence of virions surrounded by a vacuole and two double-layered membranes seems easily explainable. Presumably the vacuoles play here the same role as in the removal of foreign bodies.

In the course of the uninhibited reproduction cycle a great number of vaccinia virions are formed in each infected cell, but only few are removed and at last every infected cell dies. In methisazone-treated systems, on the other hand, virion production is less than 1% of that in the control cells and most of the infected cells survive. Obviously, a considerable proportion of the virions is kept in inclusions and thus they are removed from the cell or, at least, this process is initiated.

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EFFECT OF DIBROMOMANNITOL AND DIBROMODULCITOL ON IMMUNOLOGICAL AND MICROBIOLOGICAL SYSTEMS

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Summary. The effect of dibromodideoxymannitol (DBM) and its stereoisomer, dibromodideoxydulcitol (DBD) on immune response, growth of *Mycobacterium phlei*, and plaque formation by phlei phage have been examined. The development of anti-tetanus immunity in mice and antibody production against serum proteins in rats were inhibited by DBD more effectively than by DBM. The average survival time of skin homografts in random-bred rats was not influenced by DBM but was prolonged by two days by DBD. Although DBM was more readily soluble than DBD, the latter exerted a higher immunosuppressive effect than the former.

DBD inhibited the growth of *M. phlei* at lower concentrations than DBM. In contrast in decreasing the titre of phlei phage suspension, DBM was more effective than DBD.

Dibromomannitol (dibromodideoxymannitol, DBM) and dibromodideoxydulcitol (DBD) are α-ω-bromine-substituted six carbon atom sugaralcohols (hexitols) [7]. The formulae for the two diastereoisomer compounds are shown in Fig. 1. In screening examinations for the immunosuppressive effect of anti-tumour agents DBD proved much more active than DBM [6]. In view of this finding it seemed desirable to investigate the effect of DBD using other kinds of immunological models.

Inhibition of the division of cells involved in immunological reactions depends on several factors such as the transport, accumulation and distribution of the agent. The effect of the two diastereoisomers can be interpreted more adequately by using simple systems consisting of homogeneous cells or molecules. Accordingly, our immunological model experiments were supplemented with two microbiological models, viz. inhibition of bacterial growth and inactivation of phages.

Materials and methods

Substances. DBD (1,6-dibromo-1,6-dideoxy D-dulcitol, dibromodulcitol) is a white crystalline powder soluble in cold water at 1 : 3000. Its diastereoisomer, DBM (1,6-dibromo-1,6-dideoxy mannitol, dibromomanitol) is three times more soluble than DBD. The substances were suspended in a few drops of Tween 80 by grinding, then diluted with saline or broth to the required concentration. The substances were sterilized by UV irradiation.

For immunosuppressive treatment the agents were given in doses found effective for the treatment of refractory tumours in animals [8]. This dosage caused no considerable changes in body weight and blood counts.

Random-bred Swiss mice of both sexes weighing 32—35 g and Wistar rats weighing 180—200 g were used.

M. phlei and its specific monovalent phage were maintained on solid medium and in broth [16].

Immune response to tetanus anatoxin. Graded doses ranging from 0.04 to 5.0 combining units of adsorbed tetanus anatoxin vaccine were given in one subcutaneous injection to groups of mice. The aluminium-hydroxide-gel-adsorbed vaccine, prepared by the Institute for Sero-bacteriological Production and Research Human, Budapest, contained 10 combining units per ml.

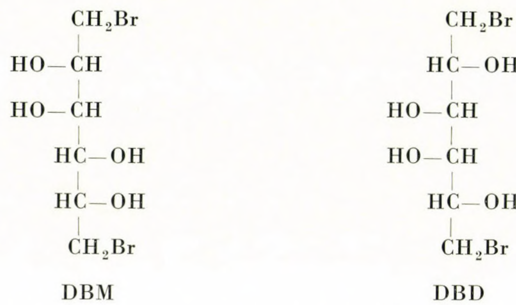

Fig. 1. Formulae for the diastereoisomers dibromomannitol (DBM) and dibromodulcitol (DBD)

Each group consisting of 30 animals was divided into 3 subgroups: (a) untreated (control) animals; (b) animals treated with DBD (300 mg/kg); (c) animals treated with DBM (300 mg/kg).

DBD and DBM were administered intraperitoneally first on the day of vaccination; subsequent injections were given twice weekly over a period of 3 weeks.

In preliminary experiments we determined the M.L.D. for fluid tetanus toxin and the lethal amount of toxin only in the group immunized with the smallest anatoxin dose. In the main experiments the animals were challenged 21 days after vaccination with the latter amount of toxin (30 M.L.D.). The ED_{50} of anatoxin was estimated by the probit test [13].

Antibody production against serum protein. Sheep, calf, rabbit, human and hen sera were mixed at equal portions and of the mixture three 2 ml doses were injected intraperitoneally at weekly intervals into 60 rats. To the first dose an equal amount of Freund adjuvant was added. The rats were divided into 3 groups each comprising 20 animals, then treated according to the following schedule: group A: untreated animals; group B: animals, treated with DBD (200 mg/kg); group C: animals treated with DBM (200 mg/kg). A total of 6 doses was given at 85 hour intervals (twice weekly). On the fourth week the animals were bled and the sera of 10 animals were mixed so that two mixed serum samples were obtained for each group. The samples were examined with the radial double diffusion technique [2] against all individual antigen sera and against their mixture. The number of precipitation lines was determined. Quantitative evaluation was made by OUDIN's linear diffusion reaction [2]. The "k" values for the albumin line of the individual antigen sera were plotted against the logarithms of the immune sera dilutions and the regression line for the points was calculated. The intersection of the regression line and the abscissa corresponded to the "equivalence point" indicating the dilution of the immune serum equivalent with the albumin concentration of the antigen serum. The equivalence points for immune sera of animals treated in different manners were compared by calculating the ratio of titres obtained in the control and in the treated animals' sera. The titration of the serum mixtures was repeated three times, then the results were analysed by STUDENT's *t*-test.

Transplantation immunity. Skin transplantation experiments on 60 rats were performed as described by BILLINGHAM and SILVERS [4]. Each animal was subjected to 2 homeo and 2 autotransplantations by the punch grafting technique [12]. The animals were divided into groups of two so that animals of one pair acted as mutual donors and recipients. After grafting the animals were divided into groups of 20. Twenty animals served as controls; the remaining two groups were selected so that each animal of all pairs fell into different groups. Three hours after grafting in one of the test groups DBM, in the other DBD treatment was started. The

drugs were administered for 3 weeks as described for immunological experiments with serum mixtures. The plaster of Paris covering applied after grafting was removed on the 8th day, then the grafts were examined daily for early (erythema, crust formation) and late (change in colour, shrinking) signs of rejection. The grafts were considered to survive until a gross change in colour and shrinking had become evident. Statistical evaluation was made by STUDENT's *t*-test.

Inhibition of bacterial growth. Stock suspensions containing 15—25 mg DBD or DBM per ml were prepared in broth. The control broth contained a minute amount of Tween 80. Then two-fold serial dilutions were made in broth and each tube was inoculated with a loopful of *M. phlei* culture. The tubes were incubated at 37 °C for 10 days and the minimal inhibitory concentrations were determined.

Inactivation of phages. One ml broth containing 10^{10} phlei phage particles was ground with 100 mg of DBD or DBM suspended in 3 drops of Tween 80. The suspension was incubated at 37 °C and sampled at intervals. Phage counts were determined in two parallel experiments by the agar layer technique [11].

Results

Inhibition of anti-tetanus immunity. Among animals not treated with the inhibitory agents the challenging dose of toxin caused 50% lethality in the group immunized with 0.15 combining units of anatoxin. In animals treated with DBM and DBD 50% lethality occurred in the groups immunized with 0.3 and 2.5 combining units of anatoxin, respectively (Table I). As concluded from the average ED_{50}, the degree of immune response was decreased by DBM about twofold, by DBD about tenfold.

Table I

Effect of prolonged DBD and DBM treatment (300 mg/kg) on the immune response of mice to tetanus anatoxin

Tetanus anatoxin (combining units) used or immun ization / Agent	5	2.5	1.2	0.6	0.3	0.15	0.08	0.04
	Number of deaths among animals challenged with 30 M.L.D. tetanus toxin subcutaneously 21 days after vaccination*							
— (control)	0/10	1/10	1/10	1/10	2/10	5/10	8/10	10/10
Dibromodulcitol (DBD)	1/10	5/10	8/10	9/10	10/10	10/10	10/10	10/10
Dibromomannitol (DBM)	0/10	0/10	1/10	2/10	5/10	6/10	10/10	10/10

* No. of deaths/No. of animals tested.

Inhibition of antibody production against serum protein. By the radial double diffusion technique immune sera of DBD-treated animals with serum mixture antigen showed only 6—7 lines. Immune sera of the control animals and DBM-treated animals produced 12 lines (Table II).

By OUDIN titration the line obtained with hen serum antigen was indistinct and could not be evaluated. The equivalence value was 1.4—2.4 times higher for control sera than for the DBD-treated animals' sera. The inhibition

Table II

Immune response to serum protein of rats receiving prolonged DBD and DBM treatment (200 mg/kg)

Immune serum prepared in rats treated with	No. of precipitation lines with antigen mixture	Decrease in the titre of sera for immunizing albumins (control animals' titre: treated animals' titre)			
		Human	Sheep	Calf	Rabbit
DBD	6—7	2.4 ± 0.2	2.2 ± 0.1	1.4 ± 0.2	1.5 ± 0.1
DBM	12	1.4 ± 0.1	1.1 ± 0.1	1.2 ± 0.2	1.0 ± 0.1
— (control)	12	Probability of the difference between the sera of control and DBD-treated animals			
		99%	99%	55%	95%

of human, sheep and rabbit albumin antibodies was significant at the 95% level. No significant difference was observed in the inhibition of anti-calf albumin antibodies. As to DBM-treated animals, the difference in equivalence value from the control sera was only 1—1.4-fold and, except for anti-human albumin, the inhibition was not significant at the 95% level. Accordingly, the two agents differed in inhibiting the immune response to various antigens. Anti-human, sheep and rabbit albumin antibodies were inhibited significantly stronger by DBD than by DBM.

Inhibition of transplantation immunity. Thirty-eight out of 40 autografts prepared in the control animals survived. The homeografts were rejected in 30 days in all animals except one in the control and one in the DBD-treated group (these were omitted from Table III). While at the dose given DBM failed to influence the survival of the grafts, DBD prolonged their life by two days on the average. The difference was significant at the 95% level (Table III).

Table III

Effect of prolonged DBD and DBM treatment (200 mg/kg) on homeograft survival in rats

Group (19—20 animals)	Average survival, days	Survival of grafts rejected earliest and latest, days
Control	12.2 ± 2.3	9—18
DBD	14.8 ± 3.3	11—20
DBM	11.8 ± 2.4	10—17

Inhibition of bacterial growth. Growth of *M. phlei* was inhibited by 218±32 μg per ml of DBD and 436±56 μg per ml of DBM. The deviations were due to growth of *M. phlei* after 5—7 days in some tubes.

Inactivation of phages. As shown in Table IV, DBM decreased the number of plaque-forming units more effectively than DBD.

Table IV

Decrease in phlei phage titre under the effect of DBD and DBM (100 mg/ml)

Agent	Phage counts × 10^7 after incubation for			
	0 hour	5 hours	10 hours	24 hours
— (control)	986±180	621±31	288±38	200±21
DBD	986±180	250±16	74±15	7±2
DBM	986±180	180±71	29±25	2±1

Discussion

Alkylating compounds constitute an important group of immunosuppressive agents. The primary site of action of these substances may be different and has not been completely elucidated [18]. The most valuable data are obtained by skin transplantation tests. The observed degree of immunosuppression depends on the method and the antigen [1, 15]. In our immunological model DBD was more effective than DBM. The degree of the inhibition of the immune response to various antigens, however, was not uniform. In our experiments the results obtained by immunizing with mixed sera should be emphasized, as certain antigens may promote the effect of one another due to eventual common components. The method used for assaying immunosuppressive activity was very instructive as it allowed a simultaneous titration of a wide scale of antigens; according to our observations not only qualitative changes (disappearance of precipitation lines) but also quantitative ones are well detected.

Biological alkylating substances, similarly to certain antimetabolites [9] and antibiotics [17] exert an influence on all cells capable of metabolism and division. Mammalian cells and bacteria, however, may considerably differ as to the effective inhibitory dose [9, 17]. In mammalian cell tissue culture DBD at 1000 μg/ml concentration inhibited growth while DBM was somewhat less effective [10]. The growth of *M. phlei* was inhibited by 1/3—1/4 of the above concentrations. Similarly to the immunological models, *M. phlei* and mammalian cell cultures are more sensitive to DBD than to DBM.

One way of studying the mode of action of inhibitory substances is the examination of their effect on molecular systems. Phage inactivation is regarded as a model for the effect of alkylating agents on DNA [18]. Radioactivity was shown in phage-DNA after treatment with labelled alkylating substances [5]. In our phage inactivation model experiments DBM exerted a stronger effect than DBD. It was questionable whether the finding was due to a difference in molecular arrangement or simply to a difference in solubility. There are several examples for the complete inactivity of the stereoisomers of active alkylating substances [14]. On the other hand, it is known that sometimes solu-

bility may influence the biological effect [3]. Our experiments with the two stereoisomers indicate that various biological models may be useful for a general study of the effect of alkylating agents.

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PRODUCTION OF ANTIBODIES AGAINST MYCOBACTERIUM PHLEI PHAGE

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Summary. The phage-neutralizing effect of spleen cells of rabbits immunized with phlei phage has been examined by phage counting and by radioactivity determination using ^{32}P-labelled phlei phages. The number of phlei phages incubated with spleen cells of the immunized animals in Parker solution decreased gradually in the cell-free supernatant during the 4 hour experiment. Actinomycin C and chloramphenicol at high concentrations suppressed the phage-neutralizing activity of immune spleen cells after 30 minutes. Immune spleen cells subjected to ultrasonic, heat and antilymphocytic serum treatment exerted phage-neutralizing activity for 30 minutes only. In the medium containing immune spleen cells treated with phlei phage free antibodies could be detected.

Previous experiments have shown that spleen cells of animals immunized with phlei phages exerted phage neutralization activity *in vitro* [4]. Neutralization was partially suppressed by actinomycin-D. From this finding it has been concluded that a two-step process is involved in the phenomenon; one step is characterized by a release of antibodies stored in the cells, the other by a synthesis of new antibodies. The present paper gives an account of investigations performed to check the validity of this hypothesis.

Materials and methods

Production of phages, immunization, serum titre and phage count determination were performed as described previously [4, 8]. The rabbits were injected four times with 2×10^{11} phages over a period of one month. For the first dose the phages were mixed to 4 ml aluminium hydroxide gel (0.5 mg $Al(OH)_3$ per ml saline) and injected intramuscularly. The second dose was given subcutaneously, the remaining two doses intravenously. Rats were immunized similarly except that they received one-half of the doses used for rabbits.

Immune spleen cell suspension was prepared as described in reference 4 with the exception that Parker solution (M 199) was used instead of Eagle's solution. The cells were washed with Hanks' solution. The suspension was divided into 3 portions: (a) untreated cells; (b) cells incubated at 56 °C for 30 minutes (trypan blue-stainable cells); (c) cells disintegrated by ultrasonic treatment in a 100 W MSE generator three times for 5 minutes.

The *in vitro* system contained 10^7 nucleated cells and 10^6 phages per ml Parker solution. Neutralization test was performed by sampling at 0, 30, 120 and 240 minute intervals. The samples were centrifuged and the supernatant was examined for phage count. The data represent the mean of two determinations.

Phage neutralization was examined in the presence of the following agents: (a) actinomycin C, 20 μg/ml; (b) chloramphenicol, 100, 500, 1000 and 2000 μg/ml; (c) antilymphocytic serum, 0.1 ml/ml incubation mixture. The antilymphocytic serum was prepared in rabbits by injecting rat lymph node homogenate. The lymph node suspension was diluted to contain

8×10^7 cells/ml. Each immunizing dose consisted of 2 ml suspension given intraperitoneally and 2 ml suspension given subcutaneously. Altogether 4 doses were given over a period of one month. Ten days after the last injection the rabbits were bled. To graded dilutions of the sera excess complement and rat lymph node cells (10^7/ml) were added. After 90 minutes incubation the cells were centrifuged and stained with trypan blue. In serum diluted 1 : 10, 90% of the cells took the stain.

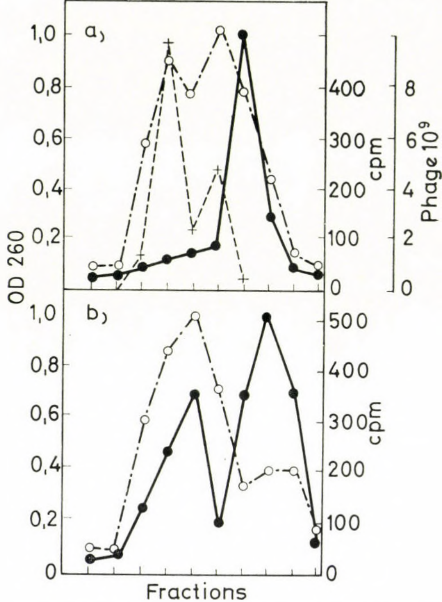

Fig. 1. Elution of phlei phage. (a) Control experiment: 0.01 μC $KH_2{}^{32}PO_4$ was added to 10^{11} phlei phages suspended in 1 ml 0.05 *M* tris buffer and the mixture was applied on Sephadex G-25 column and eluted. (b) *M. phlei*, phlei phage and 100 μC $KH_2{}^{32}PO_4$ were mixed in agar and layered in Petri dishes. After 24 hours cultivation labelled phages were prepared as described in Table I and eluted. ● —— ● = counts per minute; ○—·—·—○ = optical density at 260 mμ; +— — —+ = phage counts

Isotope experiments were carried out with phlei phages prepared by the usual method [6]. To a bacterium-phage-agar suspension sufficient for preparing 15 Petri dishes of culture, 100 μC sterile $KH_2{}^{32}PO_4$ was added. The steps of preparation are summarized in Table I. ^{32}P not incorporated in the phages was removed by passing the solution through a Sephadex G-25 column 30 ml in volume and 2 sq.cm in basal area. Elution of the phage is presented in Fig. 1. First, control experiments were performed: to 10^{11} phages in 1 ml volume 0.1 μC inorganic ^{32}P was added, then the mixture was applied on the column. The elution gave information as to the separation of inorganic ^{32}P from the phage. The number of phages was determined either by direct titration of 4 ml fractions or by measuring nucleic acid absorption at 260 mμ. The phosphorus content was indicated by the radioactive counts per minute. Separation of phage and phosphorus was not complete, but the first fractions contained phages free from inorganic phosphorus. Labelled phages were purified in a similar manner. Experiments with labelled phages were performed in a system containing 10^8 phages and 10^7 cells per ml, then the radioactivity of washed cells was estimated by means of a Gamma F 029-type scintillation counter. Detection of radioactivity was performed with "plastic phosphorus". Results were expressed in cpm/g units after subtracting the background.

Inactivated phage was prepared by alkylating dibromomannitol (Mannogranol, Chinoin, Budapest). Dibromomannitol at 250 mg amounts decreased the titre of 1 ml 10^{10} phages by 4 exponents.

In control experiments friburgensis phage [8] and spleen cell suspension of non-immunized animals were used.

Table I

Preparation of ^{32}P-labelled phlei phage
To *M. phlei* suspension (1 ml bacteria in 1 ml volume) 100 μC ^{32}P were added.
Two ml suspension and 2 ml 10^6 titrated phlei phages
were mixed into 20 ml 0.7% agar and layered into 15 Petri dishes.

Steps of preparation	Amount, ml	Number of phages per ml		Radio-activity cpm/ml	Radioactivity: phage counts observed$\times 10^{-7}$
		Calculated	Observed		
1. The agar layer culture of *M. phlei* + phlei phage mixture was extracted with broth and centrifuged at 4000 *g* for 30 minutes	40	10^{10}	10^{10}	3180	3.18
2. The supernatant was centrifuged at 35 000 *g* for 30 minutes. The precipitate was suspended in 0.05 *M* tris buffer	2	2×10^{11}	10^{11}	8700	0.87
3. The suspension was applied to Sephadex G-25 column. Fractions 3, 4 and 5 were used after elution	12	16×10^{10}	8×10^{9}	200	0.25
4. After centrifugation at 35 000 *g* for 30 minutes the precipitate was suspended in broth	1	9×10^{10}	8.7×10^{9}	210	0.24

Results

Similarly to the results of our previous studies, the number of phages incubated together with immune cells decreased gradually during the experiment. In a system containing actinomycin C, however, the phage counts remained at a constant level after 30 minutes. Cells subjected to ultrasonic or heat treatment neutralized about the same number of phlei phage particles as cells treated with 20 μg actinomycin C per ml. Chloramphenicol exerted a similar effect at 2 mg/ml doses (Fig. 2).

In the presence of antilymphocytic serum the number of phage counts decreased considerably within 30 minutes, then it showed no further change. In this experiment a spleen cell suspension of immunized rats was used. The control experiment excluded a direct action of ALS on the phages (Fig. 3).

In the above experiments, the phage count was determined in the supernatant of the incubated mixture. In subsequent experiments neutralization was studied by measuring the amount of phages bound to the cells. In 1 ml volume 10^8 labelled phages and 10^7 immune cells were incubated. After washing three times the cells showed radioactivity. When the cells were incubated with unlabelled phlei phages, washed, then reincubated with labelled phages and washed again, they showed no radioactivity. Accordingly, the labelling was considered specific for phages. The degree of radioactivity indicated the number of phages adsorbed on the cells. Phages which had disappeared from the supernatant after 15 minutes, were in their majority adsorbed on the

cells. When these cells labelled with radioactive phages were again washed three times and then transferred into Parker solution containing 10^8 unlabelled phages per ml, after 1 hour incubation the radioactivity of the cells decreased considerably (Table II).

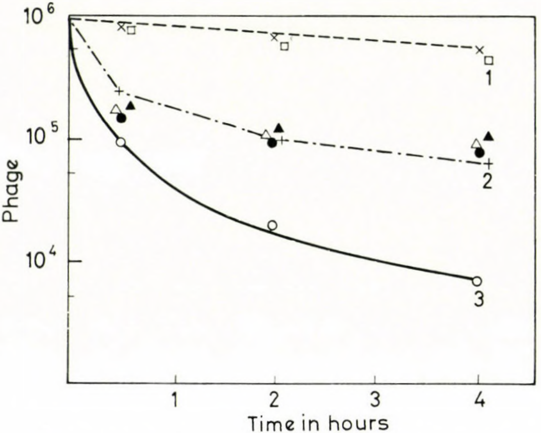

Fig. 2. Decrease in phage count under the effect of incubation with spleen treated cells by various means. Phages (10^6/ml) were incubated with cells (10^7/ml) in Parker solution. 1. Control experiment: × — — — × = cells of non-immunized animals + phlei phage; □ — — — □ = cells of immunized animals + friburgensis phage. 2. Effect of treatment: + — · — · — + = phlei phage + immune cells + chloramphenicol (2 mg/ml); ● — · — · — ● = phlei phage + immune cells + actinomycin C (20 μg/ml); △ — · — · — △ = phlei phage + immune cells inactivated by heating; ▲ — · — · — ▲ = phlei phage + immune cells subjected to ultrasonic treatment. 3. ○ —— ○ = untreated cells of non-immunized animals + phlei phage

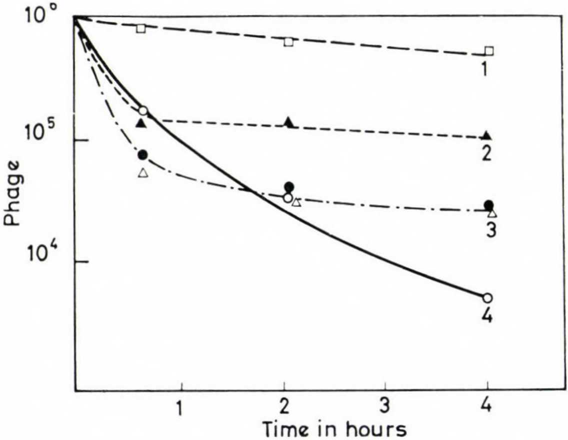

Fig. 3. Effect of antilymphocytic serum (ALS) on phage neutralization by immune spleen cells. Phages (10^6/ml) were incubated with spleen cells (10^7/ml). 1. Control experiment: □ — — — □ = cells of non-immunized animals + ALS + phlei phage. 2. ▲ — — — ▲ = immune cells subjected to ultrasonic treatment + phlei phage. 3. ○ — . — . — ○ = cells of immunized animals + phlei phage + 1/10 volume ALS; △ — . — . — △ = cells of immunized animals subjected to ultrasonic treatment + phlei phage + 1/10 volume ALS. 4. ○ —— ○ = untreated cells of immunized animals + phlei phage

Table II

Adsorption of ^{32}P-labelled phlei phage on spleen cells of immunized animals

Incubation of immune cells (10^7/ml)		Washing repeated 3 times, cells suspended in 1/10 volume	
—	After washing 3 times	Specific activity of cells (cpm/g unit); 10^{10} cells	Calculated number of adsorbed labelled phages; 10^{10} cells
1. Unlabelled friburgensis phage (10^8/ml, 15 min)	Labelled phlei phage (10^8/ml, 15 min)	201 ± 70	8×10^9
2. Unlabelled phlei phage (10^8/ml, 15 min)	Labelled phlei phage (10^8/ml, 15 min)	6 ± 73	Less than 10^8
3. Labelled phlei phage (10^8/ml, 15 min)	Unlabelled phlei phage (10^8/ml, 60 min)	80 ± 71	$\sim 10^9$

In the above experiments phages were neutralized in the presence of immune cells. The question arose as to whether neutralization occurred only on the surface of immune cells or also in the medium. In subsequent experiments the immune spleen cells were preincubated with 10^8 inactivated phages per ml for 5 minutes, then centrifuged and washed three times at 0 °C. Cells pretreated in this manner were incubated at 37 °C and then the supernatant was tested for phage-neutralizing effect. It neutralized the phages less actively than the supernatant of a system in which phages and spleen cells were incubated together throughout the experiment. The supernatant of cells incubated without phages exerted practically no neutralizing effect (Fig. 4).

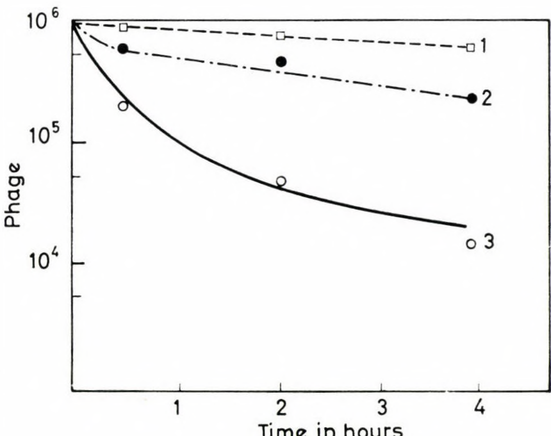

Fig. 4. **Phage neutralization by immune cell supernatants. The cell suspensions were incubated for 30, 120 and 240 minutes without phages, centrifuged and the supernatants were incubated with phlei phage (10^6/ml) at 37 °C for 1 hour. 1. Control experiment: □ ——— □ = supernatant of untreated immune cells + phlei phage. 2. ●—.—.—● = supernatant of cells preincubated with inactivated phages and washed + phlei phage. 3. Neutralization in the presence of cells: ○ ——— ○ = immune cells (10^7/ml) + phlei phage (10^6/ml)**

Discussion

The phage-neutralizing effect of immune cells damaged by heat or ultrasonic treatment indicates that the cells store antibodies which are not removed by washing. The course of phage neutralization by treated cells, unlike by whole cells, showed no graded increase during 4 hours incubation. This may have been due to the fact that the damaged cells failed to synthesize antibodies.

It was attempted to use ALS for producing a similar injury to immune cells. Remarkably, in the presence of antilymphocytic serum the degree of phage neutralization increased, a finding the interpretation of which needs further experimental data. In incubation systems containing antilymphocytic serum the cells were agglutinated in two hours and, consequently, the conditions for neutralization changed. It is known that although several antibody molecules may be bound to a single phage particle, only some of them are able to neutralize the phage. Thus not all phage particles adsorbing antibodies will be neutralized [1]. Antilymphocytic serum contains antiglobulin and therefore the precipitate produced may contain unneutralized phages. After centrifugation these are, of course, absent from the supernatant. Indicating that no antibodies were produced, neutralization did not increase after 30 minutes incubation in the system containing antilymphocytic serum.

Helmreich *et al.* [3] using a different kind of model showed that drugs inhibiting protein synthesis (e.g. puromycin) failed completely to suppress antibody production (release?). In our experiments chloramphenicol acted similarly as actinomycin C but only in very high doses. It is known that in mammalian cells *in vivo* [7] as well as *in vitro* [9] chloramphenicol inhibits protein synthesis in much higher doses than in bacterial cells.

Experiments with labelled phages indicate that phages adsorbed on the cells are later released, probably together with the antibody. The results obtained with inactivated phages may be explained by the fact that under the present experimental conditions antibody secretion did not start until the phage had come into contact with the cell. The results indicate that phage neutralization may occur on the surface of the cell as well as in the intercellular medium. Accordingly, spleen cells of animals immunized with phlei phage neutralize the phages in a manner similar to that observed with anti-MS-2 coli phage immune cells, the activity of which can be suppressed with streptomycin [5].

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TRANSFORMATION OF PHAGE RESISTANCE

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Summary. From *Bacillus subtilis* 168 M try$^-$ phs, the host strain of phage SPO-1, a spontaneous mutant has been isolated. The mutant, designated 168 M try$^-$ phr (phage-resistant), was characterized by a mutation rate of 8.32×10^{-6}. In transformation experiments the phage-resistant cells were selected as try$^+$ phr transformants.

Competence curves expressing the number of try$^+$ phs and try$^+$ phr transformants appearing at intervals during incubation were closely similar in course. The number of try$^+$ phs and try$^+$ phr transformants depended on the DNA concentration. The try$^+$ and phr characters were not linked.

During transformation the number of try$^+$ phs transformants rose rapidly to a high value after a 5 hour phenotypic lag period. Try$^+$ phr transformants appeared after 6 1/2 hours.

As compared to try$^+$ phs, the frequency of try$^+$ phr transformants was 0.12 to 0.032%.

Transformation of many characters such as antibiotic resistance, amino acid synthesis, capsule polysaccharide synthesis and sporulation, etc. has been described in various bacterial strains [1].

In the present study phage-resistant spontaneous mutants incapable of adsorbing phage SPO-1 particles were isolated from *Bacillus subtilis* strain 168 M try$^-$ phs.

A structural difference in cell wall composition has been shown to exist between phage-sensitive and phage-resistant bacteria [2]. As to the transformation of phage resistance, however, no data have been available. The present study gives an account of observations on this subject.

Materials and methods

Bacterial and phage strains. In the transformation experiments *B. subtilis* strain 168 M try$^-$ phs (sensitive to phage SPO-1) was used. The strains were kindly supplied by Dr. B. S. STRAUSS. The spontaneous mutant, showing resistance to phage SPO-1 was isolated from strain 168 M try$^-$ phs. Transforming DNA was prepared from three bacterial strains: *B. subtilis* Marburg and two prototrophic strains obtained by transformation as follows.

1. *B. subtilis* Marburg DNA $\times$ 168 M try$^-$ phs $\longrightarrow$ 168 M try$^+$ phs.
2. *B. subtilis* Marburg DNA $\times$ 168 M try$^-$ phr spontaneous mutant $\longrightarrow$ 168 M try$^+$ phr.

Culture media. The strains were maintained on potato agar [3, 4].

Pre-culturing of the recipient strain was performed on minimal glucose-yeast (MGY) agar slants. Competent cells were obtained in liquid MGY medium. Transformation was carried out in liquid T medium; the transformants were selected on MG agar. Ingredients of the media are described in reference 5.

Transforming DNA was prepared from the donor bacteria by the phenol extraction method of SAITO and MIURA [6].

Phage titration was performed by the use of our plastic plate technique [7].

Transformation. Strain 168 M try$^-$ phs was cultured on MGY agar at 37 °C overnight, then suspended in 10 ml liquid MGY medium pipetted into a 100 ml Erlenmeyer flask. The flask was fitted with a side-arm for densitometric measurements during cultivation. The culture was shaken at 100 rev/min in a water bath at 37 °C [5]. When optimal competence was reached the suspension was diluted with T medium to an optical density (OD) of 0.4. A 0.5 ml portion of the diluted suspension was added to 0.5 ml DNA diluted appropriately with T medium. The final DNA concentration was 2 μg/ml. The mixture was shaken at 37 °C for 30 minutes, then 0.2 ml of it was transferred to 10 ml MG medium containing 0.1% casein hydrolysate in a 100 ml Erlenmeyer flask. After 18 hours incubation 0.2 ml suspension (diluted with MG medium if needed) was plated on MG agar in order to select try$^+$ phs transformants. Try$^+$ phr transformants were selected as follows: 0.25 ml bacterial suspension was added to 0.75 ml phage SPO-1 suspended in MG medium to contain 3.5×10^8 particles per ml. The mixture was left to stand at room temperature for 10 minutes, then a 0.2 ml portion was plated on MG agar. The transformants were counted on the next day. Two parallel determinations were performed in each experiment.

Results

Isolation and properties of the phage-resistant mutant. In the presence of phage SPO-1 the majority of 168 M try$^-$ phs cells were killed and lysed. A few cells survived and produced colonies showing a high degree of resistance to phage SPO-1. The average phage adsorption velocity constant was 4.32×10^{-10} ml/min for the phage-resistant mutant and 4.02×10^{-9} ml/min for the phage-sensitive strain.

The following experiments were carried out to show the growth of the phage-resistant spontaneous mutant in the presence of 168 M try$^-$ phs cells. An overnight culture of 168 M try$^-$ phs cells was suspended in 10 ml MGY medium in a 100 ml Erlenmeyer flask to 0.025 OD (5.82×10^6 colony-forming units per ml). The culture was shaken in a water bath at 37 °C. After 2 hours 55 minutes, when the shortest generation time 0.2 OD had been reached, 10^9 SPO-1 phages were added to the culture. The number of phage-resistant mutants in the culture as compared to the OD of the control culture not infected with phage is shown in Fig. 1.

It is seen that the OD of the suspension decreased after infection with phage SPO-1; a subsequent increase in OD indicates the multiplication of phage-resistant cells.

In order to express the chance of mutation per bacterium per division, the mutation rate was calculated by means of Poisson's formula [8]. This value was 8.32×10^{-6}.

Ten of 168 M try$^-$ phs clones were selected. The mutation rates for these were very near to the above value.

The phage-resistant spontaneous mutant used in subsequent experiments was designated 168 M try$^-$ phr. The mutant was easily transformed to prototrophy.

Transformation of phage resistance. In these experiments (a) the competence curve; (b) the number of try$^+$ phs and try$^+$ phr transformants; and (c)

the number of transformants produced during incubation with transforming DNA were determined.

(a) The competence curve was determined as follows. Cells 168 M try$^-$ phs were cultured on MGY agar for 13 1/2 hours, then suspended in 10 ml liquid

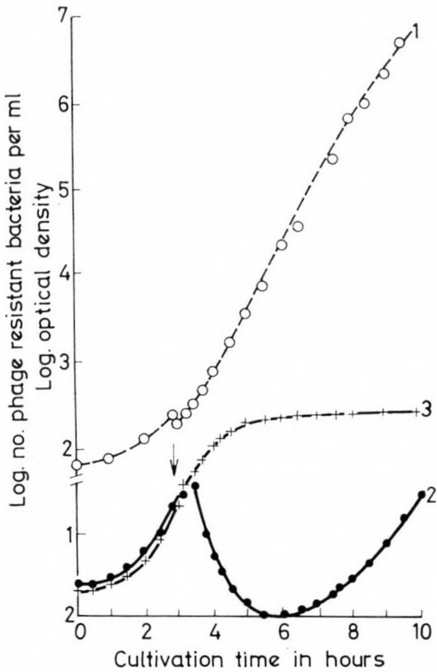

Fig. 1. Phage-resistant mutants produced in *B. subtilis* 168 M try$^-$ phs culture infected by phage SPO-1. 1 = number of phage-resistant mutants; 2 = OD of cultures infected with phage SPO-1; 3 = OD of control culture

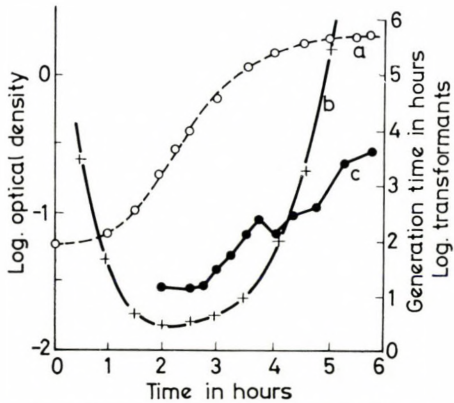

Fig. 2. Competence curve obtained from the number of try$^+$ phs transformants. a = OD; b = generation time; c = number of transformants

MGY medium. Samples taken during incubation were diluted with T medium to 0.2 OD. The diluted samples were added at 0.25 ml amounts to 0.75 ml of 168 M try$^+$ phr DNA diluted with T medium so as to give a final concentration

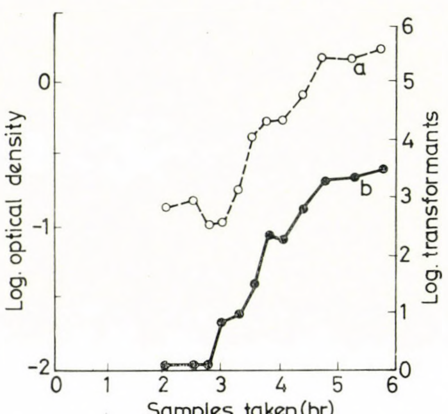

Fig. 3. Competence curve obtained from the number of try$^+$ phr transformants. a = OD; b = number of transformants

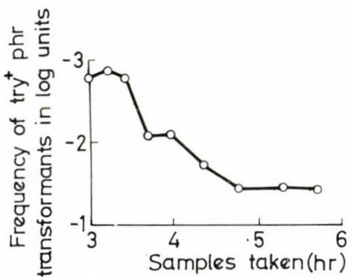

Fig. 4. Frequency of try$^+$ phr transformants during incubation

of 2 μg/ml. The mixture containing DNA + bacteria was incubated at 37 °C for 30 minutes. Then 0.2 ml of the suspension was plated on MG agar for the selection of try$^+$ phs transformants; an 0.1 ml amount was inoculated into 5 ml liquid MG medium containing 0.1% casein hydrolysate. After 18 hours incubation the phage-sensitive cells were killed by treatment with phage SPO-1 and try$^+$ phr transformants were selected on MG agar (Figs 2 and 3).

As shown in Fig. 2 the competence curve plotted from the number of try$^+$ phs transformants was similar to that obtained previously [5].

Fig. 3 shows a competence curve plotted from the number of try$^+$ phr transformants.

The initial and peak values of competence appeared at the same time, but the two curves showed no parallel course. In the initial stage of competence the number of try$^+$ phs transformants was relatively higher than that of try$^+$ phr transformants.

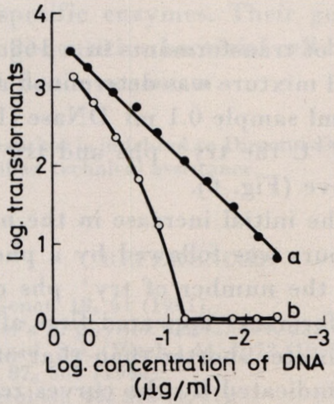

Fig. 5. Number of transformants obtained in experiments with different DNA concentrations. a = try$^+$ phs; b = try$^+$ phr

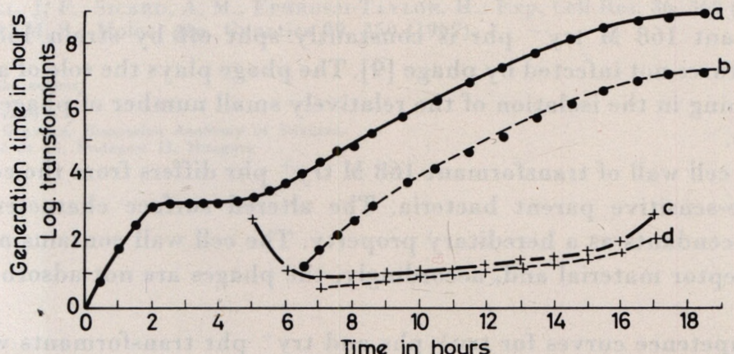

Fig. 6. Increase in the number of transformants in DNA-treated bacterial suspension. a = number of transformants for try$^+$ phs; b = number of transformants for try$^+$ phr; c = generation time for try$^+$ phs; d = generation time for try$^+$ phr

The competence curves indicated that the frequency of the two kinds of transformant varied during incubation. Fig. 4 shows the changes in the frequency of try$^+$ phr transformants.

(b) The number of transformants in competent 168 M try$^-$ phs cultures at different DNA concentrations was also determined. Transformation was performed as described above with different concentrations of 168 M try$^+$ phr DNA (Fig. 5).

The number of try$^+$ phs transformants selected in the 4th hour of transformation is characterized by a straight line. The curve for try$^+$ phr transformants selected after 8 1/2 hours incubation was not parallel with the above-mentioned straight. This finding indicates that the try$^+$ and phr markers are not linked and more than one fragment of the DNA molecule takes part in try$^+$ phr transformation.

(c) The appearance of transformants in a 168 M try$^+$ phr DNA + optimally competent bacterial mixture was determined at intervals during incubation as follows. To a 0.9 ml sample 0.1 ml DNase (10 μg) was added. After 5 minutes incubation at 37 °C the try$^+$ phs and try$^+$ phr transformants were selected as described above (Fig. 6).

It is evident that the initial increase in the number of try$^+$ phs transformants lasting for 2 hours was followed by a phenotypic lag period. Then after 5 hours incubation the number of try$^+$ phs cells showed a continuous increase. Try$^+$ phr transformants appeared first after 6 1/2 hours and their generation time was 5 minutes shorter than that of try$^+$ phs transformants. The difference is clearly indicated by the curves representing the generation times.

Discussion

Mutant 168 M try$^-$ phr is constantly split off by strain 168 M try$^-$ phs in cultures not infected by phage [9]. The phage plays the role of a selective agent helping in the isolation of the relatively small number of phage-resistant organisms.

The cell wall of transformant 168 M try$^+$ phr differs from the cell wall of the phage-sensitive parent bacteria. The altered surface character appears in the descendants as a hereditary property. The cell wall contains no specific phage receptor material and, accordingly, the phages are not adsorbed on the surface.

Competence curves for try$^+$ phs and try$^+$ phr transformants were similar in course.

The number of try$^+$ phr transformants showed no straight line relationship to the DNA concentration. This finding indicates that the try$^+$ and phr markers are not linked. Transformants with two unlinked markers were found only at saturation levels of DNA [10, 11]. Transformants with different unlinked markers are produced less frequently and even disappear in the transformation experiment performed with increasingly diluted DNA [12].

The number of try$^+$ phs transformants increased rapidly during the experiment; try$^+$ phr colonies, however, appeared in small numbers and only after 6 1/2 hours incubation. The difference was mainly due to the nature of the method used for the selection of the phage-resistant transformants. Phage

SPO-1 killed those cells in which the transformation to phage-resistance had not been completed.

The frequency of try$^+$ phr transformants, as compared to try$^+$ phs cells, was 0.12 to 0.031% after 7 hours incubation.

The reactions responsible for the biosynthesis of the phage-resistant cell wall are controlled by specific enzymes. Their genetic informations can be transformed into the phage-sensitive bacterial cell by means of DNA prepared from the phage-resistant donor organism.

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BIOCHEMICAL STUDIES ON STREPTOMYCES AUREOFACIENS

V. THE ROLE OF COBALAMINS AND METHIONINE IN METHYLATION REACTIONS IN TETRACYCLINE BIOSYNTHESIS

By

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Summary. The role of cobalamins in C-6-methyl incorporation during 7-chlortetracycline biosynthesis by *S. aureofaciens* has been studied. In media supplemented with cobalamin, *S. aureofaciens* strain C-13, which produces normally chlortetracycline and dimethylchlortetracycline in about equal proportions, failed to form dimethylchlortetracycline but showed an unaltered total tetracycline production. Cobalt ions and many other cations were ineffective; a cobamic acid derivative antagonistic to *E. coli* strain 113—3 decreased methylation activity.

Experiments using ^{14}C methionine were performed to investigate the effect of methionine on the incorporation of the 6-methyl group into the basic tetracycline structure. Methionine added at high concentration to the fermentation medium restored the methylation activity.

When the inoculum had been cultured in the presence of high concentrations of methionine, cyanocobalamin or adenine and washed before seeding, the methylation activity decreased during fermentation.

Certain *Streptomyces* strains produce 6-demethyltetracyclines [1—4] and *Streptomyces* strains forming normally 7-chlortetracycline (CTC) produce 6-dimethyl-7-chlortetracycline (DMCTC) in the presence of antimetabolites (ethionine, homocysteine analogues, D-methionine [2—7], folic acid antagonists [6], sulphonamides [5, 8, 9], norleucine [10]). These data indicate that in the tetracycline (TC) molecule C-6-methyl synthesis and methionine synthesis, that is, C_1 metabolism, are associated and that the formation of C-6-methyl and C-4-N-dimethyl groups takes place separately in tetracycline biosynthesis by *S. aureofaciens* [11, 12].

The role of methionine in the methylation of TC has been shown in studies on CTC biosynthesis [13]. It has also been demonstrated that methionine and S-adenosylmethionine take part as methyl donors in TC biosynthesis by *S. aureofaciens* and *S. rimosus* [11—14].

Investigations into the transfer of the methyl group and into methionine biosynthesis have shown that in *Escherichia coli* and *Aerobacter aerogenes* an enzyme containing cobamide (B_{12} enzyme) takes part in methylation reactions.

The effect of cyanocobalamin on DMCTC production has so far been examined only in the presence of aminopterine and ethionine. It has been found that cyanocobalamin decreases the effect of these antimetabolites [6]. Methyl-

cobalamin (α-5,6-dimethylbenzimidazolylcobamide-methyl) has recently been isolated from *S. rimosus* mycelium producing 5-hydroxytetracycline [12]. This compound is supposed to be a coenzyme of one of the enzymes — for example 5-methyl-tetrahydrofolate-homocysteine transmethylase [19] — playing an active part in transmethylation [20]. Studies on the effect of cyanocobalamin and of some cations on fermentative methylation by *S. rishiriensis* indicated that cobalt exerted a specific effect on the production of coumermycin A_1 containing a 5-methyl derivative of pyrrole-2-carbonic acid [21].

In fermentation experiments described in this paper the association between cobalamins and DMCTC—CTC biosynthesis and the effect of cobalt, cobalamins and cobalamin analogues on DMCTC production were examined. The ranges of methionine concentration influencing the DMCTC : CTC proportion in *S. aureofaciens* C-13 were also studied. Investigations performed with washed mycelium pointed to the probable role of methionine, cyanocobalamin and adenine in the regulation of methylation.

Materials and methods

Organisms. Strain C-13 was obtained from *S. aureofaciens* CDM-2114 by natural selection. Strain CDM-2114 had been produced by the use of biochemical mutants isolated previously [4]: N-4 (methionine deficient) and N6 (arginine—cysteine deficient). From these auxotrophs, which produced very small amounts of DMCTC, spore suspensions were prepared. Then both organisms were plated on the same minimal sporulation medium (Czapek-Dox) supplemented with 10^{-7} *M* L-methionine, 10^{-8} *M* L-arginine and 5×10^{-6} *M* L-cysteine, and 100 μg/ml ametopterine. After incubation at 27 °C for 14 days the developing recombinant colonies were transferred onto solid asparagine sporulation agar containing 150 μg ametopterine and 500 μg p-aminobenzolsulphonylguanidine per ml. One of the recombinants produced CTC and DMCTC at approximately equal proportions. After subjecting this culture to X-ray treatment, strain CDM-2114 was obtained. Strain C-13 was then derived from CDM-2114 by natural selection. In comparative examinations the CTC-producing strain B-28 [22] and the TC-producing strains CDSD-314 [23] and A-802934 were used. The latter was produced from CDSD-314 by the use of a chemical mutagenic agent [24].

Cultivation was carried out by shaking in corn steep liquor-starch medium or, if indicated in synthetic medium [25, 26]. Spores obtained on solid Czapek-Dox or asparagine agar were used as inocula.

For washed mycelium experiments the spores were seeded into corn steep-starch medium. The cultures were then supplemented with 5 μg cyanocobalamin, 50 μg adenine or 2280 μg L-methionine per ml and after 48 hours shaking the growth was centrifuged and washed twice in saline. Then the deposit was resuspended in 10 ml saline and used for seeding the fermentation medium. The inoculum corresponded to 33% of the volume of the original culture.

Separation of tetracyclines was performed by paper chromatography. The fermentation liquor was acidified, filtered and extracted with p-chlorphenol or n-butanol. When the antibiotics were present in low concentrations the extraction was repeated. The extracts were spotted directly on the paper sheet. After chromatography in various solvent systems the sheets were treated with ammonia and tested for fluorescence at UV light, then the CTC : DMCTC proportion was calculated [5]. Mainly two kinds of solvents were used: (a) trichloracetic acid (5%) : phosphate buffer (pH 2.0) = 25 : 5 : 20 [5]; (b) chloroform : n-butanol (saturated with aqueous 0.3 *M* H_3PO_4 and 0.1% trichloracetic acid solution) = 9 : 1 [1]. Schleicher-Schuell 2043/b and Whatman No. 1 papers were used.

Determination of chemical substances. Total tetracycline was determined after n-butanol extraction with ferric chloride reagent [22].

The concentration of biologically active cobamides was assayed by the agar diffusion test using *E. coli* mutant 113—3 [27] and *Lactobacillus leichmanii* strain ATCC 7830 [28] after boiling the fermentation liquor with cyanide.

The cobalamin antagonist used in the experiments was first shown by KELEMEN and SIMON as a natural metabolite of *Propionibacterium shermanii* [29, 30]. It corresponded to α-5,6-dimethylbenzimidazolylcobamic acid-*a,b,c,d,g*-pentaamide-cyanide (DCAPA). The antivitamin effect of this substance was assayed with *E. coli* mutant 113—3 [29, 30].

Microbiological assay of methionine was performed turbidimetrically with *E. coli* mutant 133—3. The disturbing effect of cobalamins was eliminated with DCAPA [31].

Isotope examinations were carried out by methods described previously [26]. Activity (counts per minute) was calculated for 1 mg tetracycline antibiotic.

Results

Strains C-13, B-28, CDSD-314 and A-802934 were compared for total cobamide, total TC and DMCTC production by fermentation for 4 days in a medium containing 1 mg cobalt ions per ml.

Table I shows that total TC and DMCTC production varied considerably, but there was no significant difference in the concentration of cobamides.

Table I

*Total TC, DMCTC and cobalamin production by S. aureofaciens strains**

Strains	Total TC μg/ml	DMCTC μg/ml	DMCTC per cent	Total cobalamin μg/ml	
S. aureofaciens C-13	1950	880	45	3.2**	1.8***
S. aureofaciens B-28	940	0	0	2.4	1.4
S. aureofaciens CDSD-314	1720	0	0	3.6	2.0
S. aureofaciens A-802934	4100	0	0	3.1	1.9

* Each figure represents an average of 6 experiments.
** Assayed as cyanocobalamin with *E. coli* 113—3.
*** Assayed as cyanocobalamin with *L. leichmanii*.

In subsequent fermentations the effect of cobalt ions and other cations on cobamide biosynthesis and fermentative methylation [21] was examined. Results obtained for strains C-13 and B-28 (Table II) indicate that cobalt ions at the concentration used exerted no action on fermentative methylation, cobamide synthesis and total TC synthesis [32]. Other ions (Zn, Mg, Mn, Al, Cu, Fe, Pb) at 0.1—100 μg/ml concentration in synthetic medium [26] also failed to influence methylation.

Table II presents data for the effect of cyanocobalamin and hydroxycobalamin as well as for the supposed inhibitory effect of cobinamide cyanide ("B factor") on methylation [33].

From Table II it is evident that cobalamins and even cobinamide at concentrations as low as 0.1 μg/ml considerably influenced the fermentative methylation in strain C-13; DMCTC production, with a practically unaltered total TC production, decreased by 40%. When cobalamin or cobinamide was

Table II

Effect of cobalt ions, cyanocobalamin and hydroxycobalamin on fermentative methylation

Additions at zero time		Strain C-13				Strain B-28		
		Total TC μg/ml	DMCTC μg/ml	DMCTC per cent	Total cobal-amin μg/ml*	Total TC μg/ml	DMCTC per cent	Total cobal-amin μg/ml*
Co^{++} 0.1 μg/ml		1860	840	45	3.0	910	0	2.8
Co^{++} 5.0 μg/ml		1770	710	40	3.4	1050	0	2.5
Co^{++} 10.0 μg/ml		1710	770	45	3.2	870	0	2.9
Cyanocobalamin	0.1 μg/ml	1720	80—170	5—10	.	850	0	.
,,	1.0 μg/ml	1840	0	0	.	810	0	.
Hydroxycobalamin	0.1 μg/ml	1790	80—180	5—10	.	930	0	.
,,	1.0 μg/ml	1740	0	0	.	860	0	.
Cobinamidocyanide	0.1 μg/ml	1710	80—170	5—10	.	900	0	.
,,	1.0 μg/ml	1800	0	0	.	780	0	.
,,	10.0 μg/ml	1680	0	0	.	750	0	.
,,	50.0 μg/ml	1510	0	0	.	700	0	.

* Assayed as cyanocobalamin with *E. coli* 113—3.

applied at 1.0 μg/ml concentration, strain C-13 produced only the 6-methyl derivative, CTC.

The effect on methylation of cyanocobalamin added at different intervals during fermentation is presented in Table III. After 24 hours or later the agent was practically ineffective. When added at the 16th hour of fermentation, the parallel experiments showed considerably varying results as to the propor-

Table III

*Effect of cyanocobalamin on fermentative methylation**

Time of addition	Total TC at time of addition, μg/ml	At end of fermentation		
		Total TC μg/ml	DMCTC μg/ml	DMCTC per cent
—	—	1760	790	45
0 hr.	0	1810	0	0
8 hr.	40	1880	0	0
16 hr.	120	1780	80—450**	5—25**
24 hr.	280	1820	640—820**	35—45**
36 hr.	520	1880	850	45

* Cyanocobalamin concentration, 1.0 μg/ml.
** Range of DMCTC concentration in parallel experiments.

tion of DMCTC; nevertheless, the inhibition of DMCTC synthesis was still marked.

The specific effect of cobalamins was proved by adding DCAPA [29, 30]. The cobalamin antagonist was added at different concentrations at the beginning of fermentation. As shown in Table IV, DCAPA influenced not only fermentative methylation but also total TC biosynthesis. In addition, it decreased mycelium production. Although DCAPA significantly decreased total TC biosynthesis, in strain C-13 there was a considerable shift in the proportion of DMCTC. In the culture of strain B-28 DMCTC could be detected, but, curiously enough, the demethyl analogue of TC was absent from cultures CDSD-314 and A-802934.

Table IV

Effect of DCAPA on fermentative methylation

Strains	Additions								
	DCAPA, 10 μg/ml			DCAPA, 40 μg/ml			DCAPA, 80 μg/ml		
	Total TC μg/ml	DMCTC μg/ml	DMCTC per cent	Total TC μg/ml	DMCTC μg/ml	DMCTC per cent	Total TC μg/ml	DMCTC μg/ml	DMCTC per cent
C-13	1510	680	45	720	430	60	400	340	85
B-28	780	0	0	480	0—50	0—10	310	30	10
CDSD-314	1240	0	0	620	0	0	370	0	0
A-802934	3100	0	0	1850	0	0	680	0	0

The effect on C-6-methylation of other cobalamin antagonists was also examined. Simple molecules as benzimidazoles and purines have been described as weak cyanocobalamin antagonists [28]. In the present experiments 5,6-dimethylbenzimidazole, adenine, guanine and xanthine exerted no effect on methylation by any of the strains tested.

When a washed inoculum which had been precultured in the presence of cyanocobalamin at high concentration was used, the biosynthesis of DMCTC increased, that is, methylation decreased. It was striking that DMCTC was produced if a certain amount of cyanocobalamin had been added to the medium (Table V). Similar results were obtained when the inoculum was cultured in the presence of adenine. At the end of the fermentation DMCTC appeared in a somewhat increased proportion but total TC synthesis remained well below the usual level (Table V) and mycelium production also decreased.

Under similar conditions strains B-28, CDSD-314 and A-802934 produced neither DMCTC nor 6-demethyl-TC.

Similar experiments were carried out with guanine, xanthine and with uracil. DMCTC production was influenced only by the purines, but to a lesser degree than by adenine.

Table V

Effect of cyanocobalamin and adenine on fermentative methylation in C-13 cultures started from washed mycelium

Additions to inoculum medium	Additions to fermentation medium					
	—			Cyanocobalamin, 0.1 μg/ml		
	Total TC μg/ml	DMCTC μg/ml	DMCTC per cent	Total TC μg/ml	DMCTC μg/ml	DMCTC per cent
—	1820	820	45	1720	80—170	5—10
Cyanocobalamin, 5 μg/ml*	1510	750	50	1500	680	45
Adenine, 50 μg/ml*	540	350	65	.	.	.

Additions to inoculum medium	Additions to fermentation medium					
	Cyanocobalamin, 1.0 μg/ml			Adenine, 50 μg/ml		
	Total TC μg/ml	DMCTC μg/ml	DMCTC per cent	Total TC μg/ml	DMCTC μg/ml	DMCTC per cent
—	1780	0	0	1610	720	45
Cyanocobalamin, 5 μg/ml*	1610	640	40	.	.	.
Adenine, 50 μg/ml*	.	.	.	720	500	70

* The inoculum was cultured in the presence of cyanocobalamin or adenine by shaking for 48 hours and washed before seeding.

Among the possible methyl-donor and methyl-precursor amino acids the role of methionine, serine and glycine was examined. In the presence of methionine, *S. aureofaciens* C-13 produced DMCTC in smaller amounts, that is, methylation activity increased and in extreme cases only CTC could be detected in the culture (Table VI). Serine (in the presence or in the absence of pyridoxine) and glycine exerted no influence on fermentative methylation.

C-13 spores were inoculated into a liquid medium containing 2400 μg L-methionine per ml. After incubation the culture was washed and seeded into corn steep-starch medium. At the end of the fermentation a DMCTC concentration was somewhat increased (Table VII). The methylation activity remained unaltered when L-methionine was added in lower concentrations (Table VI). At higher methionine concentrations only CTC was produced. It was interesting that the effect of methionine was eliminated by very low concentrations of cyanocobalamin. Similarly to cyanocobalamin and adenine, methionine exerted no influence on methylation in strains B-28, CDSD-314 and A-802934.

In subsequent experiments a washed inoculum cultured in the presence of high methionine concentrations was seeded into synthetic medium. Mycelium production and DMCTC : CTC proportion are shown in Fig. 1. Total DMCTC

Table VI

Effect of amino acids on DMCTC production by strain C-13

Additions*		Total TC µg/ml	DMCTC µg/ml	DMCTC per cent
—		1700	770	45
L-methionine	150 µg/ml	1650	500	30
,,	500 µg/ml	1780	80—180	5—10
,,	1000 µg/ml	1620	0	0
L-serine	1000 µg/ml	1600	640—800	40—50
Glycine	1000 µg/ml	1640	640—820	40—50

* Corn steep medium containing 40 µg/ml of methionine was supplemented at seeding with the substances indicated.

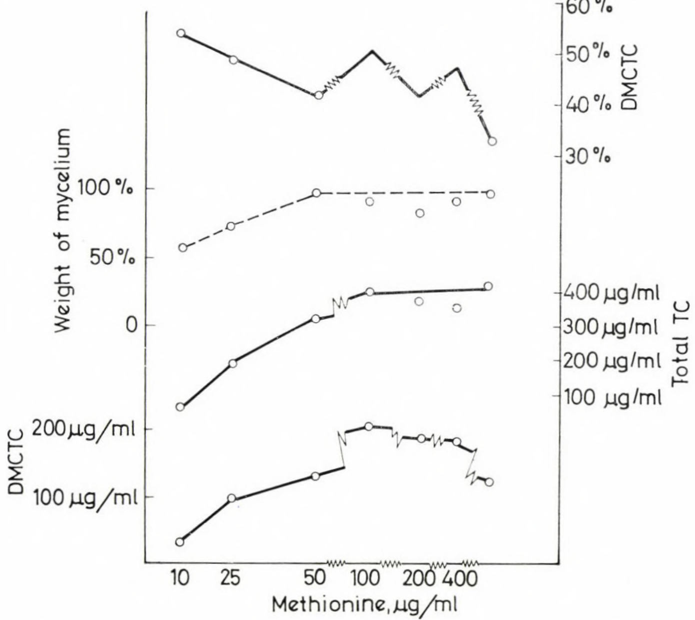

Fig. 1. Mycelium, total TC and DMCTC production in synthetic medium. The inoculum was cultured in the presence of methionine (2380 µg/ml) by shaking for 48 hours and washed before seeding

concentrations calculated from the percentage values and total TC contents are also presented.

Although the standard error and analytical difficulties of the experiment are considerable, it may be concluded that in respect to DMCTC production an optimal methionine concentration exists. When the methionine con-

Table VII

Effect of methionine on fermentative methylation in C-13 cultures started from washed inoculum

Addition to inoculum medium*	DMCTC per cent after fermentation in basal medium** supplemented with				
	—	L-methionine			Cyanocobalamin 1.0 μg/ml
		150 μg/ml	500 μg/ml	1000 μg/ml	
—	45	30	5—10	0	0
L-methionine 2280 μg/ml	60	50	35	0—5	0

* The basal inoculum medium contained 100 μg methionine per ml. The inoculum was cultured by shaking for 48 hours and washed before seeding.
** The basal fermentation medium contained 40 μg methionine per ml.

centration is increased, the production of DMCTC decreases although the total TC production may increase.

Table VIII summarizes our experiments with ^{14}C-labelled methionine. When strain C-13 was cultured in the presence of ^{14}C-L-methionine, then paper-chromatographed and eluted [26], CTC radioactivity calculated for 1 mg TC was 50% higher than DMCTC radioactivity. This finding indicates that the methyl donor in strain C-13 is L-methionine (S-adenosylmethionine) for both C-6-methyl and C-4-N-dimethyl groups. Accordingly, methionine plays an equal role in the incorporation of all three methyl groups into the basic tetracycline structure.

Similar results were obtained when the above experiment was performed with washed inoculum cultured in the presence of methionine at high concentrations (the DMCTC : CTC radioactivity corresponded to 2 : 3). Values obtained for 1 mg CTC and DMCTC were also similar for both kinds of culture (Table VIII).

Accordingly, there was no difference in the incorporation of the three methyl groups when a washed inoculum produced in a medium containing methionine at high concentrations was used. The differences in DMCTC synthesis may be explained by a disturbance in the synthesis of the methyl donor. Paper chromatography in chloroform—butanol solvent of cultures started from washed inocula which had been grown in the presence of methionine at high concentrations revealed a spot characterized by low radioactivity and bluish fluorescence. The spot, situated between the start line and the TC spot ($R_f = 0.12-0.15$), was named "DM factor". The radioactivity of one unit of DM factor was estimated as 20—50% of CTC. Probably the same DM factor was detected in C-13 and B-28 cultures fermented in the presence of p-amino-benzolsulphonylguanidine [5]. In cultures of some recombinants producing DMCTC at low total TC levels probably the same DM factor was demonstrated at high concentrations. These recombinants had been obtained from methio-

Table VIII

Incorporation of the methyl group of methionine into CTC and DMCTC in C-13 cultures started from washed mycelium

Addition to inoculum medium*	Addition to fermentation medium**									
	4 μc labelled methionine					4 μc labelled methionine + methionine 500 μg/ml				
	Total TC μg/ml	DMCTC μg/ml	DMCTC per cent	Counts per min per mg		Total TC μg/ml	DMCTC μg/ml	DMCTC per cent	Counts per min per mg	
				CTC	DMCTC				CTC	DMCTC
—	1510	680	45	3410	1980	1600	0	0	3580	—
L-methionine, 2280 μg/ml	1280	640	50	3240	2150	1150	350	30	3850	2420

* The basal inoculum medium contained 100 μg methionine per ml. The inoculum was cultured by shaking for 48 hours and washed before seeding.
** The basal fermentation medium contained 40 μg methionine per ml.

nine—cysteine auxotrophs as described for strain DCM-2114 under Materials and Methods. The DM factor has not been identified; it probably corresponds to an N-demethyl derivative of DMCTC.

Discussion

It is known that the C-6-methylation of the basic tetracycline structure requires folic acid [5, 6]. Results presented in this study indicate that the methylation reaction is associated with a "B_{12} enzyme".

The C-6-methylation reaction was perfectly restored in strain C-13 by culturing in the presence of cobalamins and cobinamide; the culture produced only CTC. Guest and Woods [33] showed that in *E. coli* cobinamide inhibited the biosynthesis of methionine from serine and homocysteine. The present study revealed no similar process, *i.e.* a decreased methylation at C-6 for either of strains C-13 and B-28. The failure to inhibit methylation may be explained by the activation of cobinamide or by the inability of cobinamide to serve as a methyl acceptor in *S. aureofaciens*. In contrast, cobinamide restituted methylation activity in strain C-13, that is, the culture produced no DMCTC in the presence of that substance.

Cobalt ions and other cations exerted no effect on methylation and, accordingly, unlike in coumermycin A_1 biosynthesis by *S. rishiriensis*, cations failed to stimulate enzymes taking part in this process. Although the degree of cobalamin permeability is not known for *S. aureofaciens*, it would appear that permeability is not such an important factor in strain C-13 as it has been supposed to be in *S. rishiriensis* [21].

The time course of the elimination of C-6-methylation inhibition can adequately be followed by adding cyanocobalamin at various intervals during fermentation. This substance was ineffective after 36 hours. The explanation is probably that the polyketide structure [11] was transformed into an intermediary product unable to serve as a methyl acceptor at C-6. A less likely explanation is that the transformation of cyanocobalamin to active cobamide form cannot take place in aged cultures. An interesting but easily explainable phenomenon is the considerable difference in DMCTC synthesis between parallel cultures supplied with cyanocobalamin after 16 hours. The difference is due probably to the condition of the culture and the progress of TC biosynthesis. The fact that after the development of the mycelium, that is, after primary metabolism had come to an end cobalamin was ineffective on C-6 methylation, indicates that the biosynthesis of a secondary metabolite, TC, is closely associated with primary metabolism.

Experiments with DCAPA show also that a "B_{12} enzyme" may play an important role in the general metabolism of, and in C-6 methylation reaction in, *S. aureofaciens*. Although in 2 out of the 4 TC-producing strains the 6-demethyl analogue of TC could not be detected, the effect of DCAPA was evident from the decreased TC synthesis and mycelium production also in these strains. The result is noticeable because the antagonistic action of DCAPA has so far been shown only for *E. coli* mutant 113–3.

The role of "B_{12} enzymes" may not be restricted merely to the transfer of methyl groups but, as pointed out by BUCHANAN *et al.*, a "B_{12} enzyme" taking part in methionine biosynthesis may actively regulate the concentration of various folates [17, 35]. The role of "B_{12} enzymes" in the 6-methylation reaction for the basic TC structure may be associated with its direct role in methionine biosynthesis.

On supplementing with methionine the fermentation medium of strain C-13, the methylation activity at C-6 increased and at high methionine concentrations only CTC was produced. This finding may be analogous to the phenomenon observed with methionine-auxotroph mutants [2] when in the presence of a constant methionine level DMCTC was produced at the end of the fermentation. Unfortunately, it has not yet been studied why the C-6 methylation activity decreases in the case of a relative methionine deficiency while formation of the C-4-N-dimethyl group on the TC precursor is taking place probably completely. It may be supposed that the enzyme responsible for C-6 methylation is much less active than the enzyme facilitating C-4-N-methylation or that the methyl donor must be present at higher concentrations for C-6 methylation than for C-4-N methylation. This consideration may explain the behaviour of prototrophic C-13 strain, during the fermentation of which C-6 methylation activity was restituted with methionine. It may also be supposed that — similarly to *E. coli* in which homocysteine methylation may be facilitated

by two enzyme mechanisms (one associated with "B_{12} enzyme" and the other with a folic acid pathway) — in *S. aureofaciens* also different enzyme mechanisms are involved in C-6 and in C-4-N methylation.

Quantitative changes in DMCTC production observed with washed inocula grown in the presence of relatively high concentrations of cyanocobalamin, adenine and methionine may be due to disorders in the regulation mechanism influencing the synthesis of the methyl donor. Because of a relative methyl donor deficiency the C-6 methylation reaction decreases in activity or a higher methionine concentration is needed for the complete restitution of C-6 methylation. Further studies involving the preparation of a suitable enzyme are needed to elucidate whether this finding is a consequence of an alteration in the regulating mechanism of methyl donor synthesis and whether the phenomenon is an enzyme repression.

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COMPARATIVE STUDIES ON THE BIOLOGICAL PROPERTIES OF ESCHERICHIA COLI O124 : K72(B17) STRAINS

By

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Summary. A total of 725 *E. coli* O124 : K72(B17) strains have been examined for lysogenicity, colicinogenicity, flagellar antigens and lactose fermentation. The strains have been divided into 4 groups and 7 types by means of a combined phage-colicin typing scheme. Colicinogenic strains occurred more frequently than lysogenic cultures. Type 1 (colicinogenic) strains were the commonest among the isolates. Type 2 (lysogenic) strains differed from the rest in fermenting lactose more rapidly and being uniformly non-flagellated. The majority of motile strains bore H antigen 30. Some strains in type 4 contained H antigen 27 or undetermined H antigens.

Differentiation of colicins and phages released from the strains was performed by heat, chloroform, pH and trypsin sensitivity testing.

Lysogenicity experiments with type 1b (lysogenic + colicinogenic) strains have indicated that under natural conditions these organisms are derived from type 1 (colicinogenic) and type 4 (lysogenic) strains.

Escherichia coli serogroup 124 strains were first isolated by Ewing and Gravatti in 1944 [1]. During the past 24 years a number of foreign [2—12] and Hungarian [13—24] authors isolated the organism from water and food-borne outbreaks and sporadic cases and investigated its epidemiological and bacteriological properties.

In view of the frequent incidence of *E. coli* O124 : K72(B17) (hereafter *E. coli* O124) strains in Hungary, the lysogenic properties of this organism have extensively been investigated in our institute and a typing scheme based on the lysis spectrum of *E. coli* O124 temperate phages has been elaborated [16, 22, 23].

Further studies showed that the inhibitory action of *E. coli* O124 cultures on the indicator strains was frequently not due to the lytic action of phages as a considerable number of the cultures released agents which could not be propagated and when diluted, they failed to produce plaques on plates seeded with the indicator strains. These findings indicated that cultures behaving in this manner produced colicins.

Hamon *et al.* [25] elaborated various physical, chemical and biological tests for the differentiation of phages and colicins. They described pH and proteolytic enzyme sensitivity tests as the most important differential reactions: at pH 3 phages, in contrast to bacteriocins, were highly damaged or

completely inactivated while to proteolytic enzymes the bacteriophages were resistant and bacteriocins of Gram negative gelatin-non-liquefying bacteria were sensitive. Chloroform, which was well tolerated by the phages, inactivated bacteriocins produced by many Gram negative organisms.

According to BRANDIS [26] phages and colicins may act on the same receptors. Our findings have confirmed this conception as some indicator strains for *E. coli* O124 temperate phages have been found sensitive to both lethal agents.

Our studies on a great number of strains have shown that colicinogenic cultures can be characterized and divided into types on the basis of their inhibitory effect on a set of indicator strains used in lysogenicity testing and on *E. coli* strain Row, an international detector of colicinogenicity.

As the overwhelming majority of colicinogenic strains were identical in inhibition spectrum, *i.e.* they belonged to the same colicin type, further colicin-sensitive indicator strains have been introduced. Strains of ABBOTT and SHANNON's set described for the typing of *Sh. sonnei* [27] were sensitive to colicins released from *E. coli* O124. Accordingly, in order to be able to distinguish a greater number of types, all strains were tested with the two sets of indicator cultures.

Previous studies [22] have shown that *E. coli* O124 strains isolated from various foci differ in the time of lactose fermentation. On the basis of acid production in 1% lactose the strains could be divided into 2 groups. Strains belonging to one of these groups produced acid more rapidly (after 2 to 3 days) than members of the other group which fermented this sugar less readily (after 4 to 17 days). As there was a definite association between the time of lactose fermentation and the lytic spectrum of the strains, the question arose as to whether a similar association existed with flagellar antigens. Accordingly, it seemed desirable to supplement the observations with the determination of H antigens.

Materials and methods

E. coli O124 strains. A total of 725 strains were selected. Most of them were isolated in various areas of Hungary from outbreaks, sporadic cases and carriers. Some cultures were obtained from Czechoslovakia (Dr. E. ALDOVÁ), Roumania (Dr. I. D. COSTIN) and Japan (Dr. H. MATSUMOTO).

Detection and differentiation of phages and colicins. Indicator strains described in [22] were used: *E. coli* B, *E. coli* 36, *Sh. sonnei* II, *E. coli* 138 and 218 (O124), *Sh. flexneri* 262, FREDERICQ's strain derived from *E. coli* K12, *E. coli* Row and a set of indicator strains described by ABBOTT and SHANNON [27] for the typing of *Sh. sonnei.*

Temperate phages and colicins were obtained by growing *E. coli* O124 strains in pH 7.2 Hartley broth at 37 °C overnight. Typing was carried out on Hartley agar plates.

Lysogenicity and colicinogenicity were examined as described by PAPAVASSILIOU [29]. The broth cultures were centrifuged and the supernatants were treated with 1/10 part of chloroform. A number of cultures were treated parallel with heat and chloroform, then two-hour broth cultures of the indicator strains were mixed at 0.5 ml aliquots into 2 ml soft nutrient agar and layered over agar plates. When the layer had hardened, demarcated zones were prepared by impressing a sterile glass tube 15 mm in diameter into the agar and the treated supernatants

were spotted onto them. In this manner 23 supernatants could be tested on one plate. The plates were read after 5 hours incubation at 37 °C, then left to stand at room temperature overnight and read again.

In the comparative examination of colicin typing the method of FREDERICQ [30] and the indicator strains of ABBOTT and SHANNON [27] were employed.

Inhibition patterns obtained with the indicator strains were designated according to HART's scheme [28].

Differentiation of phages and colicins was performed by examining their sensitivity to trypsin, pH, heat and chloroform as described by HAMON *et al.* [25].

Lactose fermentation. The strains were cultured on lactose-eosin-methylene blue medium, then inoculated into a liquid medium containing Bacto peptone (Difco), 10 g; NaCl, 5 g; lactose, 10 g; Andrade indicator, 10 ml; distilled water, 1000 ml.

Serological examinations. Immune sera were prepared from *E. coli* type strains obtained from the State Serum Institute, Copenhagen. Type strain *Sh. dysenteriae* 3 (Large-Sachs Q 771) was received from the collection of the Institute of Microbiology, University Medical School, Pécs. O and OK sera were prepared as described in the literature [31—33]. H antigens for immunization, absorption and agglutination were prepared as follows. The cultures were passaged several times on semisolid agar at 37 °C or at 30 °C [34], then inoculated onto swarm agar plates. After suspending the growth in saline, formalin was added and the density was adjusted. For slide agglutination dense suspensions were prepared (one loopful mixed into one drop of serum gave a suspension optimal for slide test). For tube agglutination the suspension was adjusted so as to correspond to a standard giving 74% transmittance in 1 : 10 dilution in 1 cm cuvettes of the Beckman model DU spectrophotometer set at 0.03 mm slit and 530 mμ wavelength; tube agglutination was performed with 0.5 ml serum dilutions and 0.05 ml bacterial suspensions.

H antigen determination was performed by slide agglutination; 165 strains were examined also by the single tube agglutination technique using sera diluted 1 : 500 or 1 : 1000 according to the titre of the serum. Tube tests for O, K and H antigen determination were read uniformly after 18 to 20 hours incubation at 37 °C. This technique yielded no significant differences in comparison to the 48—50 °C incubation described in the literature.

H sera were prepared in addition to the type strains from some of our isolates containing H antigens 30 and 27 or H antigens not determinable with H type sera.

With the exception of the known H30—H32 relationship, no remarkable H cross reactions were revealed between the H type antigens. H30 and H32 sera were cross-absorbed before use. Strains not containing antigen H30 and cultures giving weak or atypical agglutination were checked by immobilization test with the homologous serum. Immobilization was examined microscopically in broth cultures or in U tubes containing H sera mixed in semisolid-agar [31, 35, 36].

Results

Determination of colicin and phage types. The combined phage-colicin typing schema developed on the basis of our previously described method, H antigens and lactose fermentation reactions of the types are presented in Table I.

On the basis of lysogenicity and colicinogenicity the strains were divided into 4 groups. Within the 4 groups the strains fell into 7 types according to their spectrum of inhibition on the indicator strains. Group I contained lysogenic, group II colicinogenic and group III lysogenic + colicinogenic strains. Cultures exhibiting neither lysogenic nor colicinogenic property were classified into group IV.

Table II shows the group and type distribution of the examined strains. Out of 725 cultures 246 (33.9%) belonged to group I, 342 (47.2%) to group II, 28 (3.9%) to group III and 109 (15.0%) to group IV. Type 1 (colicinogenic) and type 2 (lysogenic) strains were frequently encountered (42.8 and 25.8%, respectively).

Table I

Classification of E. coli O124 strains on the basis of lysogenicity, colicinogenicity, H antigens and lactose fermentation

Group	Type	Lysis or inhibition spectrum						H antigen	Lactose fermentation, days	
		E. coli 36	*Sh. sonnei* II	*E. coli* 138	*E. coli* 218	*Sh. flexneri* 262	*E. coli* Row		2–3	4–17
I	2	±	+	±	±	—	—	—	+	
	4	—	—	+	±	—	—	+		+
	6	—	—	—	—	+	—	d		+
II	1	×	—	—	—	—	×	+		+
	2b	×	×	×	×	×	×	+		+
III	1b	×	—	+	±	—	×	+		+
IV	Nt	—	—	—	—	—	—	d		+

+ = lysis or positive character
± = irregularly positive or negative
× = inhibition
d = flagellated or non-flagellated

Table II

Distribution of E. coli O124 strains according to the combined phage-colicin scheme

Group	Type	Strains No.	Strains %	Group	Type	Strains No.	Strains %
I	2	187	25.8	II	1	310	42.8
					2b	32	4.4
	4	41	5.6	III	1b	28	3.9
	6	18	2.5	IV	Nt	109	15.0

Typable strains, 616 (85.0%).
Untypable strains, 109 (15.0%).
Total, 725 (100.0%).

Differentiation of phage and colicin activity. A total of 50 *E. coli* O124 strains belonging to various types were selected. In accordance with the finding of Hamon *et al.* [25] it was observed that temperate phages released from lysogenic strains were more sensitive to heating than colicins. While the phages were resistant to chloroform, colicins showed a marked sensitivity to this substance: the colicin titre was lost in one strain and considerably decreased in 86% of the remaining 28 colicinogenic strains. Hydrogen ion concentration sensitivity testing showed that the phage titre began to decrease at pH 5 and

became nil at pH 3. No decrease in colicin activity was observed at pH 4 and 5; at pH 3 the colicin titre in two cultures decreased but was not entirely lost. Trypsin treatment exerted no effect on temperate phages but inactivated colicins.

Although our experiments did not include all known tests for the differentiation of phages and colicins, the above findings have been considered satisfactory for establishing our combined *E. coli* O124 typing scheme.

Comparative examination of colicin-typing. A total of 370 colicinogenic *E. coli* strains were examined for inhibition spectrum on indicator strains of our and ABBOTT and SHANNON's sets (Table III).

Table III

Distribution of E. coli O124 strains according to colicinogenic properties

HART's scheme	Present scheme			Total No. of strains
	1	1b*	2b	
3	—	—	12	12
3A	—	—	13	13
6	310	27	7	344
11	—	1	—	—
Total	310	28	32	369

* lysogenic + colicinogenic

It was shown that 344 strains (93.0%), including type 1 and 1b cultures of our scheme, fell into HART's type 6. One type 1b strain, a colicinogenic culture, inhibited the growth of only strain Row. Thirty-two strains classified into our type 2b fell into 3 different colicin types of the HART scheme. Epidemiological studies, however, failed to show any association between foci and HART's 3 types.

Examination of 80 colicinogenic strains isolated from 13 different small outbreaks indicated that whenever our typing method revealed a difference between strains originating from the same focus, a similar difference was detected by typing according to HART's scheme. For example, in a family outbreak affecting 8 persons 7 patients excreted type 1, but from one member of the family type 2b was isolated by several times repeated examinations. According to HART's scheme all strains but our type 2b belonged to type 6. The latter corresponded to HART's type 3.

Serological examinations. Out of the examined 725 *E. coli* O124 strains 512 were flagellated. With the exception of 9 strains all flagellated isolates were characterized by H antigen 30. Type strain *E. coli* O124 227 described by EWING as containing H antigen 32 [33] was also found to bear H antigen 30.

Strain 227, which had been received from the State Serum Institute, Copenhagen, was compared with *E. coli* N 157, type strain for H antigen 30. Cross-absorption experiments indicated that the two strains contained identical flagellar antigens. In subsequent antigenic analyses it was shown that *E. coli* O124 strain 916 of LÁNYI *et al.* [15], which had been isolated from an outbreak and described as identical in antigenic structure with strain 227, contained also flagellar antigen 30. In order to elucidate this problem we had forwarded our data to DR. W. H. EWING.

It is worth mentioning that two *E. coli* O124 strains not included in the Tables contained H antigen 12. These cultures had been obtained from Japan and were untypable by our scheme.

Nine strains containing flagellar antigens other than H30 were mostly lysogenic. Eight of them lysed indicator strain *E. coli* 218 and were therefore classified into type 4. The remaining one strain was neither lysogenic nor colicinogenic. As to O and K antigens these cultures differed in partial antigens from EWING's strain 227, but showed no significant antigenic relationships to other O and K antigens of the *E. coli* antigenic scheme.

Five of the above 9 strains contained H antigen 27. The remaining 4 strains were not related in H antigens to the available 46 *E. coli* H antigen type strains (H 1—12, H 14—21, H 23—48); according to the findings these strains contained 3 different, probably new H antigens.

Lactose fermentation. In agreement with our previous studies, the present experiments with 725 strains showed that lysogenic cultures belonging to type 2 produced acid in 1% lactose within 72 hours (Table I). Other lysogenic and non-lysogenic strains fermented lactose more slowly. The time of lactose fermentation was checked with 834 colonies of 86 *E. coli* O124 strains belonging to various types. Testing of 339 colonies of 26 type 2 strains in lactose-peptone water resulted in 338 positive tubes within 3 days; only one subculture produced acid after 4 days. Most subcultures of other types fermented lactose after 6 to 9 days. Apart from some differences due to the size of the inoculum, the two biogroups could well be distinguished. It should be noted that while lactose fermentation in 2 to 3 days was a constant property characteristic of type 2 strains, many exceptions were noted among the "slow-lactose-fermenting" cultures, especially in the colicinogenic group.

Discussion

Types determined on the basis of lysogenic and colicinogenic properties of *E. coli* O124 are characterized as follows.

The *lysogenic group* (group I) can be divided into 3 types. *Type 2* strains differ from other members of group I in exerting a smaller degree of lytic action on several indicator strains, lysing *Sh. sonnei* II, being non-flagellated and

fermenting 1% lactose after 2 to 3 days. In the lysogenic group type 2 occurred the most frequently.

Temperate phages of *type 4* strains lyse either *E. coli* 138 or *E. coli* 218 or both. Type 4 may therefore be subdivided on the basis of lytic action on the two indicator strains; in view of the low incidence of these strains, the subdivision is not of practical value. All strains in type 4 were flagellated and, with the exception of the mentioned 8 strains lysing *E. coli* 218, contained H antigen 30. All of them fermented lactose after a prolonged period of time.

Unfrequently occurring *type 6* strains lyse *Sh. flexneri* 262, ferment lactose slowly and may or may not be flagellated.

The *colicinogenic group* (group II) contains two types. *Type 1*, the commonest among *E. coli* O124 strains, is characterized by inhibiting *E. coli* 36 and *E. coli* Row. The colicin of *type 2b* usually inhibits the growth of all indicator cultures. Both types are late lactose fermenters and flagellated.

Strains belonging to *type 1b* are *lysogenic and colicinogenic* (group III). These cultures inhibit the growth of *E. coli* 36, produce plaques on *E. coli* 138 and 218, ferment lactose slowly and are flagellated.

On the basis of the lysis spectra it was supposed that type 1b strains had developed from type 1 colicinogenic and type 4 lysogenic organisms. Lysogenization experiments have confirmed this theory: when a type 1 colicinogenic strain was treated with phages released from type 4, the resulting culture (type 1b) showed both lysogenic and colicinogenic properties.

It may be concluded from these findings that in the majority of *E. coli* O124 strains the lysogenic and colicinogenic characters are not linked and only few isolates possess both properties.

Group IV comprises strains which are neither lysogenic nor colicinogenic and cannot therefore be divided on the basis of these characters.

The examined strain collection was selected so as to represent all types with a sufficient number of isolates. Accordingly, the incidence of types presented in Table II does not reflect their real distribution in nature. In an unselected material collected over a period of one year the distribution of 486 *E. coli* O124 isolates was: lysogenic strains, 26%; colicinogenic strains, 46%; lysogenic + colicinogenic strains, 4%; non-lysogenic, non-colicinogenic strains, 24%. Thus colicinogenic strains occurred more frequently than lysogenic cultures.

In comparing HART's scheme and our colicin typing method we found that, in respect to *E. coli* O124, the former does not yield better results than those obtained with the latter method. An advantage of our scheme is that it permits the simultaneous determination of lysogenic and colicinogenic characters in *E. coli* O124.

The suitability of our scheme for subdividing colicinogenic *E. coli* O124 cultures has been confirmed by the following facts. International type strain

MLE+ producing colicin E_1 and our strains belonging to type 1 showed identical inhibition patterns on the indicator strains. *E. coli* strain 235 producing colicin E_1 + I was identical in inhibitory spectrum with our type 2b strains. Three of our colicinogenic isolates differed somewhat from both type 1 and type 2b in inhibiting the growth of *E. coli* B, 36 and Row. According to the HART scheme, these cultures belonged to type 3 and in inhibitory spectrum they were identical with type strain *E. coli* 230 producing colicin V + E_1. In view of their infrequent incidence these strains were omitted from Table II.

Comparative examinations indicate that colicinogenic *E. coli* O124 strains release 3 kinds of colicin.

As in *E. coli* O124 the colicin titre decreases after chloroform treatment, for combined colicin-phage typing it is advisable to heat the culture supernatants for the destruction of bacteria. Heating at 56 °C for 30 minutes is not injurious either to phages or to colicins.

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STUDIES ON SWINE SERUM IMMUNOGLOBULINS

I. PROTEIN FRACTIONS IN SWINE SERUM

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Summary. Immunoelectrophoreses with high precipitin titre rabbit antisera revealed 28 components in swine serum. The following fractions were shown: albumin, 3 prealbumins, $7\alpha_1$-globulins, $5\alpha_2$-globulins, $5\beta_1$-globulins, 4 (+1) β_2-globulins and 2γ-globulins. One of the β_1-globulin fractions was probably identical with transferrin. IgG, IgA and IgM appeared distinctly among the globulin fractions.

Previous experiments using free electrophoresis [15] showed quantitative changes in the serum protein components of immunized pigs. For studying qualitative changes the immunoelectrophoresis technique [7] was chosen.

On the basis of differences in antigenicity about 30 protein components have been distinguished in human serum [1, 6]. Studies on a number of mammals have revealed more than 20 different protein fractions in the serum of each species. There are few data as to the composition of swine serum. Brummerstedt-Hansen showed 24 protein components in healthy, adult pigs [2]. Lecce *et al.* in studying the difference between newborn and adult pigs' sera and changes occurring during growth found 19 protein components in fullgrown pigs [9—11]. Kim, Bradley and Watson examined the development of immunoglobulins of piglets [8]. Franek *et al.* studied a special gamma-globulin present in newborn piglets [3, 4].

As no agreement has been reached in the literature concerning the number and classification of protein fractions, we regarded the elucidation of the composition of swine serum as an important step prior to studying immunoglobulins in detail. The present paper describes immunoelectrophoretic studies of swine serum.

Materials and methods

Swine sera. Untreated animals and animals immunized to high antibody content with viral and bacterial antigens in the course of routine serum production were examined. Blood samples taken from the anterior *vena cava* were left to coagulate and the clear serum obtained after the retraction of the clot was used. The serum samples were examined as a rule within 1 to 3 weeks; the tests were sometimes repeated after several months. The sera were preserved with 400—500 units per ml of penicillin and 0.4—0.5 mg of streptomycin and stored at 2—4 °C.

Precipitating sera. Well-developed rabbits were injected with whole swine serum. In order to promote the antigenicity of some minor components the serum was given with an adjuvant: to 6 parts liquid paraffin 1 part Tween 80 was mixed and with 5 parts swine serum an emulsion was prepared. To 36 ml emulsion 40 mg killed *Mycobacterium tuberculosis* were added.

Nine doses each consisting of 2 ml antigen were injected subcutaneously at weekly intervals. The rabbits were bled under general anaesthesia 8 days after the last injection. The serum was sterilized by filtration, preserved with penicillin and streptomycin as described for swine serum, distributed at 2—3 ml portions and stored in the refrigerator.

The rabbits tolerated the injections well except for necrosis at the site of the injections.

Two rabbits were injected with normal swine serum, 2 with high titre anti-swine erysipelas and 6 with anti-hog cholera serum.

Immunoelectrophoresis. SCHEIDEGGER's micromethod [14] was somewhat modified in that each slide was layered with 4 ml gel prepared with 2% agar in pH 8.6 borate buffer at 0.025 ionic strength. Then at a distance of 33 mm from the anode-side end of the slide antigen wells 3.0 mm in diameter and about 15 cu.mm in volume were prepared.

The current was adjusted to 5 V/cm and electrophoresis was performed for 2 to 2 1/4 hours. Then longitudinal troughs 2 mm in width were cut in the agar at 3.5 mm distance from the antigen well. According to the aim of the experiment either two different swine sera were pipetted into two wells and one rabbit serum sample into a trough out along the middle of the slide or a central well was filled with one swine serum sample and two peripheral troughs with two different rabbit sera.

The patterns were allowed to develop for 40 hours in a moist chamber, then the free proteins were washed out and the agar layer was dried. The precipitation lines were stainded with 0.1% acid fuchsin dissolved in 10% glacial acetic acid. Excess dye was removed by washing several times with 5% glacial acetic acid solution.

Results

By means of the described immunization method, very high titre precipitating sera were obtained. Tube tests showed that the swine sera gave precipitation with the rabbit sera at 10^{-6} and sometimes at 10^{-7} dilution. In view of the purpose of this study the capacity of the sera to react with a large number of swine serum components was more important than the precipitation titre. Summarized data for all rabbit sera tested have shown that swine serum contains 28 protein components.

In each of the individual rabbit sera less than 28 precipitins were revealed. The exact determination of their number is difficult. The production of precipitins is influenced not only by the composition of swine serum used as antigen but also by the individual reactivity of the immunized rabbit. Thus low titre precipitins produce but occasionally well-detectable reactions.

The importance of the relative quantity of protein components in the swine serum antigen became clear when the detection of immunoglobulins was attempted. The sera of rabbits immunized with normal pig sera were unsuitable for the detection of IgA and IgM fractions. In contrast, rabbit sera prepared with the sera of pigs immunized to high antibody content gave distinct reaction with these immunoglobulins.

The results with individual rabbit sera are subject to the physico-chemical laws of immunodiffusion reactions [1]. Thus the dilution of the examined swine sera was successfully applied in order to obtain a distinct reaction for protein fractions the precipitation line of which would otherwise be redissolved in consequence of the antigen excess. Precipitation lines for protein components present in high concentrations showed a shift to an unusual site; this phenomenon could also be prevented by diluting the swine serum.

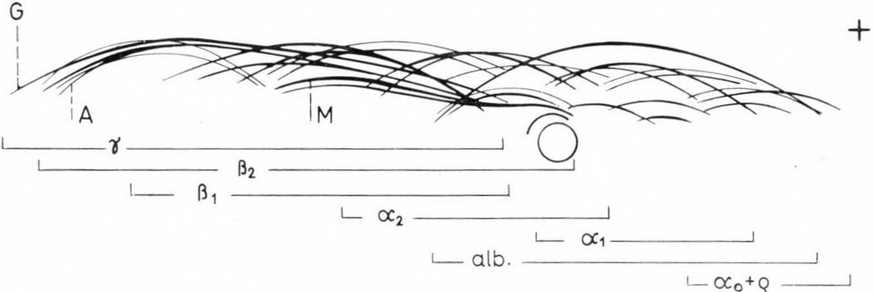

Fig. 1. Schematic representation of protein fractions in swine serum. Lines G, A and M are immunoglobulins

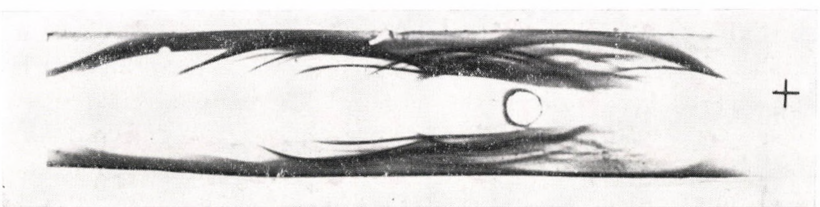

Fig. 2. Immunoelectrophoretic pattern of anti-hog cholera swine serum examined with two different rabbit sera

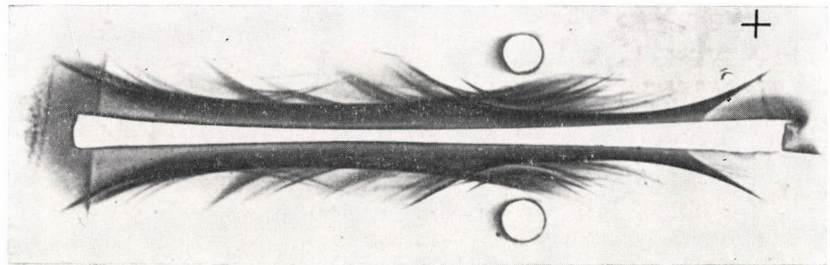

Fig. 3. Immunoelectrophoretic pattern of two different anti-hog cholera swine sera examined with rabbit immune serum

In view of the above observations it is evident that the detection of all components in a whole serum cannot be expected in one immunoelectrophoresis experiment, as optimum conditions for all antigen-antibody reactions hardly prevail in a single system. Accordingly, the complete pattern of swine serum protein components can only be determined if several experimental results are summarized. Fig. 1 shows a schematic diagram of all fractions revealed. In Fig. 2 and Fig. 3 the original reactions of various protein groups are presented. Fig. 2 indicates all fractions detected in the alpha-globulin group and more rapidly migrating beta-globulin components. Fig. 3 presents

in detail precipitation lines obtained for the slower $beta_2$ and gamma-globulin groups.

The data indicated the presence of 28 immunologically different components in swine serum. In the prealbumin group 3 protein components ($alpha_0$ and ϱ fractions) were shown. In the $alpha_1$-globulin group migrating at about the same speed as albumin 7 fractions were differentiated. These components all produced precipitation lines within the albumin arc.

The distinct and uniform albumin line reached from the anode-side end of the pattern as far as beyond the serum reservoir. With diluted swine sera a fine, sharp line running parallel with the albumin arc was sometimes observed.

The $alpha_2$-globulin group contained 5 fractions located by the cathode-side end of the albumin arc.

In the $beta_1$-globulin group 5 fractions were distinguished. There were two faint arcs situated closely beyond the $alpha_2$ group; 3 more distinct fractions appeared farther back toward the cathode. The largest, most distinct arc positioned nearest to the rabbit serum reservoir corresponded probably to the transferrin component of human sera. This fraction appeared in all swine sera at the same location and always as a strong, distinct precipitin line. Under this line there was a fraction characterized by the flattest arc revealed in this group; this component was probably the $beta_1$ C globulin.

Under the $beta_1$-globulin group there was an almost straight line corresponding to IgM ("M" in Fig. 1). In the serum of pigs immunized to high antibody content this fraction appeared as a very distinct, elongated, slightly bent, doubly arched line reaching toward the anode as far as the serum reservoir or even somewhat beyond it. With some rabbit immune sera this fraction appeared as two parallel closely spaced arcs.

Another important member of group $beta_2$ is IgA ("A" in Fig. 1). A large part of its arc shaded into the IgG line; next to the cathode the two lines separated and the IgA arc ended in the concave part of the IgG line. At the same site there were two faint lines the highly convex arcs of which run approximately parallel with each other and the anode-side parts of which intersected the more rapidly migrating beta-fractions. The inner of these fractions might correspond to human $beta_2$ C, the outer to $beta_2$ S globulin fraction, but it cannot be excluded that they are immunoglobulins.

In swine serum, as in human serum, IgG produces the longest line ("G" in Fig. 1). Starting from the serum reservoir it passes as a practically straight line through the space of beta globulins, then ends toward the cathode in a bent arc representing the slowest fraction. The IgG arc often shows a double ending. With some rabbit antisera it appeared in the form of two separated parallel lines.

Discussion

From the present findings it may be concluded that all components corresponding to the important human serum fractions can be detected in swine serum. The final identification of these fractions needs further investigations. As the purpose of the present work was to study immunoglobulins in detail, in respect to other serum fractions it is sufficient to summarize the results of immunoelectrophoretic examinations.

In connection with the description of various serum fractions it should be mentioned that in our experiments the prealbumin arc appeared distinctly. BRUMMERSTEDT-HANSEN supposed that this fraction existed but, probably owing to its weak antigenicity, he failed to demonstrate it [2]. The present findings indicate that our immunization method is suitable for preparing effective precipitating sera against very weakly antigenic fractions or against components present in very small amounts. The occasional faint line running parallel with the albumin arc may similarly be interpreted. It may be supposed that for this rare phenomenon the sensitive detection of allotype albumin molecules was responsible.

The doubling of IgG and IgM lines indicates also antigenic differences between molecules belonging to these fractions. In agreement with the finding of FUDENBERG and FUDENBERG [5] for human gamma-globulin, RASMUSSEN [13] demonstrated the presence of combinations of two isoantigens in swine serum. METZGER and FOUGEREAU [12] showed two subclasses in swine gamma-G-globulin which differed in the composition of the heavy peptide chains. The doubling of the cathode-side end of the IgG arc in the present experiments may be attributed to a successful demonstration of these two subclasses. In contrast, the parallel double arc extending over the whole length of the line can be regarded as a reaction given by different light peptide chains. This double line was observed only with some rabbit sera which always gave double IgG arcs for all swine sera. This result indicates that individual sera may contain alternatively one or the other kind of light peptide chains within the molecules of any Ig class. The occasional doubling of the IgM arc is due probably to the existence of different subclasses. This reaction was not demonstrated for all sera and may, consequently, be attributed to individual differences. The joining of the two double arcs for IgM and IgG, which would indicate an antigenic identity, was never observed. This finding also confirms that the doubling of the IgM arc was not due to the detection of light peptide chains.

Among the globulin fractions shown the three most important immunoglobulins, IgG, IgA and IgM (G, A and M in Fig. 1) can well be recognized. The association between antibodies and IgG is well known. The relationship of IgA and IgM fractions to antibodies in swine serum will be the subject of a subsequent paper.

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Phylaxia State Serum Institute,
Szállás u, 3, Budapest X, Hungary

INDEX

Tomus XVI

Fasciculus 1

Merétey, K., Várterész, V., Elekes, E.: Immunological Activity of Ribonucleoproteins. II. Immune Diffusion Studies of Ribonucleoproteins Extracted from Guinea Pig Liver ... 1

Merétey, K., Várterész, V., Kutas, V., Kocsár, L.: Immunological Activity of Ribonucleoproteins. III. Changes in Mammalian Ribonucleoproteins upon the Effect of Whole Body X-irradiation ... 11

Fodor, F., Kocsár, L., Lendvay, J., Merétey, K., Tapfer, D., Vedres, I.: Persistence of Human Gamma-globulin in Animals ... 19

Lázár, G.: Effect of Dibromomannitol on the Dextran-induced Increase of Local Capillary Permeability and on the Anaphylactoid Reaction ... 27

Bartha, T., Mérő, E., Szita, J.: Bacteriological Model Experiments on Radiosterilization of Catgut ... 31

Samir, M. A.: Studies on Peritoneal Exudate of Animals Experimentally Infected with *Toxoplasma gondii.* I. Effect of Heat on Activity ... 39

Lovas, B., Hollós, I.: Replication of Vaccinia Virus in the Embryonated Egg in the Presence of Methisazone. An Electron-microscopic Study ... 47

Elek, G., Földes, I., Vandra, E.: Effect of Dibromomannitol and Dibromodulcitol on Immunological and Microbiological Systems ... 63

Elek, G., Vandra, E., Földes, I.: Production of Antibodies against *Mycobacterium phlei* Phage ... 69

Horváth, S.: Transformation of Phage Resistance ... 77

Járai, M.: Biochemical Studies on *Streptomyces aureofaciens.* V. The Role of Cobalamins and Methionine in Methylation Reactions in Tetracycline Biosynthesis ... 85

Deák, S., Ádám, M. M.: Comparative Studies on the Biological Properties of *Escherichia coli* O124 : K72(B17) Strains ... 97

Surján, J.: Studies on Swine Serum Immunoglobulins. I. Protein Fractions in Swine Serum ... 107

The Journal of General Microbiology

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R. C. LANCEFIELD Current problems in studies of Streptococci
E. G. HUBERT, C. S. POTTER, G. M. KALMANSON and L. B. GUZE Pigment formation in L-forms of *Serratia marcescens*
D. M. POWER and S. W. CHALLINOR The effects of inositol-deficiency on the chemical composition of the yeast cell wall
F. D. CALONGE, A. H. FIELDING and R. J. W. BYRDE Multivesicular bodies in *Sclerotinia fructigena* and their possible relation to extracellular enzyme secretion
G. J. DRING and A. HURST Observations on the action of benzylpenicillin on a strain of *Streptococcus lactis*
K. SEN, P. NANDI and A. K. MISHRA Transformation of nutritionally deficient mutants of *Aspergillus niger*
D. T. KINGSBURY, G. R. FANNING, K. E. JOHNSON and D. J. BRENNER Thermal stability of interspecies *Neisseria* DNA duplexes
M. M. ATTWOOD An investigation into the mode of action of actinonin
A. D. ANTOINE and B. S. TEPPER Environmental control of glycogen and lipid content of *Mycobacterium phlei*
V. BOFFI Biochemical patterns of some heterotrophic marine bacteria grown in defined media
T. W. K. YOUNG Electron and phase-contrast microscopy of spores in two species of the genus *Mycotypha* (Mucorales)
P. NORBERG and B. V. HOFSTEN Proteolytic enzymes from extremely halophilic bacteria
G. MARZULLO and W. F. DANFORTH Ethanol-soluble intermediates and products of acetate metabolism by *Euglena gracilis* var. *bacillaris*
D. W. HOLLOMAN Biochemistry of germination in *Peronospora tabacina* (Adam) Conidia: evidence for the existence of stable messenger RNA
R. M. CONNAMACHER Specificity of phenotypic adaptation of *Bacillus cereus* to tetracycline
D. A. STAFFORD and A. G. CALLELY The utilization of thiocyanate by a heterotrophic bacterium
A. J. CLUTTERBUCK Cell volume per nucleus in haploid and diploid strains of *Aspergillus nidulans*
M. PIOTROWSKA, M. SAWICKI and P. WEGLENSKI Mutants of the arginine-proline pathway in *Aspergillus nidulans*
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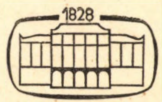

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EFFECT ON INFLUENZA VIRUS OF A MODIFIED FRANCIS INHIBITOR AND ITS ACETONE-SOLUBLE FRACTION

III. EXPERIMENTS ON THE MOUSE LUNG

By

I. Hollós

National Institute of Public Health (Director: T. Bakács*), Budapest*

(Received June 8, 1968)

Summary. Francis inhibitor (K_0), its diethyl-p-phenylene diazonium salt derivative (*8m*) and the acetone-soluble fraction (*8ac*) of the latter were examined for inhibitory action on the multiplication in the mouse lung of an influenza A-1 virus strain. The substances were administered intraperitoneally. *8m* and *8ac* proved to be active against 50 ID_{50} and 800 ID_{50} of virus, respectively. Inhibition of infections with larger doses needed toxic doses of *8ac*. Inhalation of the ether-soluble fraction of *8ac* was inhibitory even if the inoculum was as large as 5×10^5 ID_{50}.

Some chemical and physico-chemical properties of the substances have been determined. The neuraminic acid content and the rate of its liberation by viral enzyme seemed to be characteristic features.

In previous experiments [1, 2, 3] the diethyl-p-phenylene-diazonium salt derivative (*8m*) of the Francis inhibitor (K_0) and, particularly, the acetone-soluble fraction (*8ac*) of *8m* significantly inhibited the multiplication of influenza virus in both surviving chorio-allantoic membrane fragments and de-embryonated eggs [4, 5]. Five mg per ml medium of *8ac* proved to affect events taking place in the reproduction cycle between 30 minutes and 2 hours, 7.5 mg per ml of the same preparation appeared to influence also later events of the cycle. Still larger doses (20 mg per ml) of *8ac* stopped the cycle and liberated virions from the surface.

In the present study the preparations were examined for antiviral effect in the mouse lung. As test virus, an influenza A-1 virus strain was used.

The substances were subjected to analytical ultracentrifugation, and their nitrogen, neuraminic acid and lipid contents were determined.

Materials and methods

Preparation of Francis inhibitor, the statistical methods applied and the titration of influenza virus in Horváth's rolling drum were described in a previous report [4].

Virus. The strain influenza A-1 Budapest 4/49 had undergone 113 intranasal passages in white mice of 16—18 g body weight, obtained from the breed of this Institute. The virus multiplied in the lungs of the mice but failed to kill them. Passages were performed at 72-hour intervals. The lung suspension obtained from passage 113 was lyophilized. For the present experiments the lyophilized virus was resuspended, and lung suspensions from passage 114 were used as inocula throughout.

Inoculation of mice. Mice under light ether anaesthesia were inoculated by intranasal instillation of virus in a volume of 0.05 ml per mouse. After 20 minutes or more the mice were anaesthetized again and each was given 0.05 ml of saline in the same way.

Antiviral treatment. Infected mice were immediately divided into groups at random. Each mouse was injected intraperitoneally several times with 0.5 ml of antiviral solution or saline. The injection schedules and the treatment of mice in Experiments III and IV are given under Results.

Chemical assays. Nitrogen was determined by a Kjeldahl micro-method, lipid phosphorus and total lipid according to Fiske and Subba Row and gravimetrically, respectively. Cholesterol was extracted as described by WEBSTER [6] and estimated with the Liebermann—Burghard reagent as described by TUZSON *et al.* [7].

Free and fixed neuraminic acid was determined by WARREN's [8] method as modified by AMINOFF [9]. To determine fixed neuraminic acid, samples were hydrolyzed at 80 °C in 0.1 *N* H_2SO_4. The optimum period of hydrolysis was 20 minutes.

Enzymatic hydrolysis was performed with the PR8 strain of influenza A virus at 37 °C in the presence of 2.0% substrate. In the reaction mixture the haemagglutination titre of the virus was 1 : 24, the period of digestion was 24 hours.

To determine free (or liberated) neuraminic acid, 0.1 ml of 0.2 *M* sodium metaperiodate dissolved in 9 *M* solution of phosphoric acid was added to 0.2 ml of the sample to be tested. The mixture was allowed to stand at room temperature for 20 minutes, then it was shaken with 1 ml of a 10% solution of sodium arsenite containing 0.1 *N* H_2SO_4 and 0.5 *M* Na_2SO_4. The mixture thus obtained was shaken and 3 ml of a solution of 0.6% thiobarbituric acid in 0.5 *M* Na_2SO_4 was added. The tube was supplied with a glass cap, placed for 15 minutes in boiling water and subsequently in melting ice. From each tube a 4.3 ml sample was shaken with an equal volume of butanol containing 5% concentrated hydrochloric acid, and the mixtures were centrifuged for 3 minutes. The coloured supernatants were examined in a Uvifot photometer at 546 mμ wave length. Results were expressed in μg of N-acetylneuraminic acid per 0.2 ml.

The extinction curve was calculated from a series of experiments in which neuramine lactose was hydrolyzed with sulphuric acid.

Analytical ultracentrifugation. The analytical rotor of the MOM G 120 ultracentrifuge and the Philpot—Svensson optics were used. $S_{W20°}$ was calculated on the basis of the moving boundary method [10].

Inhibitor derivatives were centrifuged at 50,000 or 60,000 r.p.m. and photographs were taken at 9- or 12-minute intervals.

Results

Experiment I. Mice were infected with 50 ID_{50} of virus. Subsequently the animals were injected intraperitoneally seven times, 1, 8, 15, 24, 31, 38 and 48 hours after infection. The total dose per mouse of K_0, *8m* and *8ac* was 7×6.4, 7×6.4 and 7×5.0 mg, respectively. Control mice received 7×0.5 ml of saline. From every group four mice were decapitated at 24, 48, 72 and 96 hours after infection. The lungs of mice of the same history were pooled. Ten per cent lung suspensions were centrifuged for 15 minutes at 3000 r.p.m. and a 3.16-fold (0.5 log unit) series of dilution was prepared from each suspension. From every dilution 0.1 ml was measured into each of four tubes of HORVÁTH's roller drum. Titre was calculated by the REED—MUENCH formula.

K_0 failed to elicit inhibition. Inhibition by *8m* and *8ac* is shown in part A of Fig. 1. The two curves run below the control curve, approximately together, except at 24 hours when the inhibitory effect of *8ac* was more pronounced.

Experiment II. In this experiment the apparently ineffective K_0 was omitted and 800 ID_{50} of virus was used as inoculum. Otherwise the experimental conditions were the same as in Experiment I.

The results are shown in Part B of Fig. 1. Owing to the larger inoculum, the inhibition was less conspicuous than in Experiment I. The curve illustrating virus multiplication in *8ac*-treated mice ran slightly below the corresponding curve for *8m*.

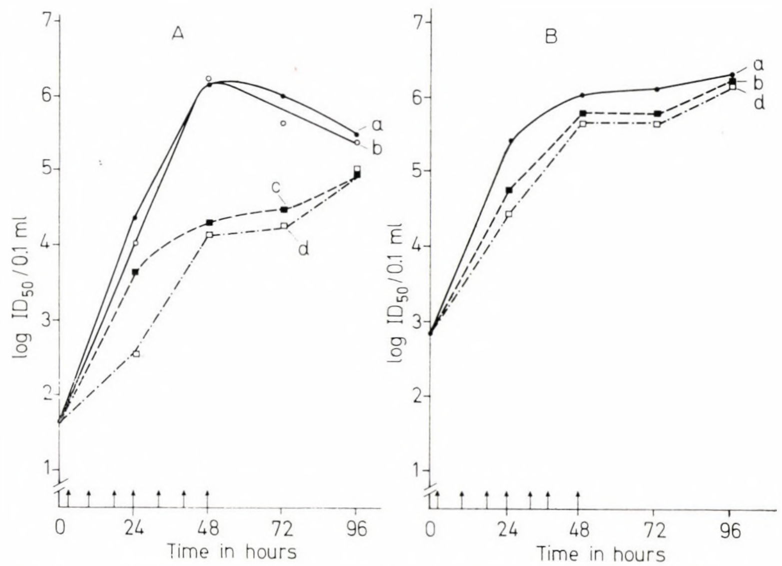

Fig. 1. Inhibition of influenza virus multiplication in the mouse lung. Strain: A-1 Budapest 4/49. Inoculum: A: 50 ID_{50}, B: 800 ID_{50}. Explanation: a = Control; b = K_0; c = *8m*; d = *8ac*. Arrows indicate the time of intraperitoneal injections. For further explanations, see the text

For statistical analysis, Experiments I and II were combined. To prove the existence of virus inhibition, the sign test was applied. In the presence of *8m* the virus titre was lower than in the control system or in the presence of K_0 in every case but one. Thus, $p = 9 \times 2^{-8} < 5\%$. For *8ac*, $p = 2^{-6} < 5\%$. Inhibition was therefore significant for both substances.

Experiment III. In this experiment we attempted to administer *8ac*, *i.e.*, the preparation that had appeared the most effective, through the respiratory tract. In view of the technical difficulties and the experience described under Discussion, we applied the following procedure.

One and a half gram of *8ac* was suspended in 120 ml peroxide-free ether and the suspension was poured into a cylindrical 2-litre flask. Mice were infected with 10^3 ID_{50} of virus and then put in the 2-litre flask, on a perforated plate over the ether-suspended *8ac*. The mice were kept there until light anaesthesia had set in. The procedure was repeated in each of the first 15 hours

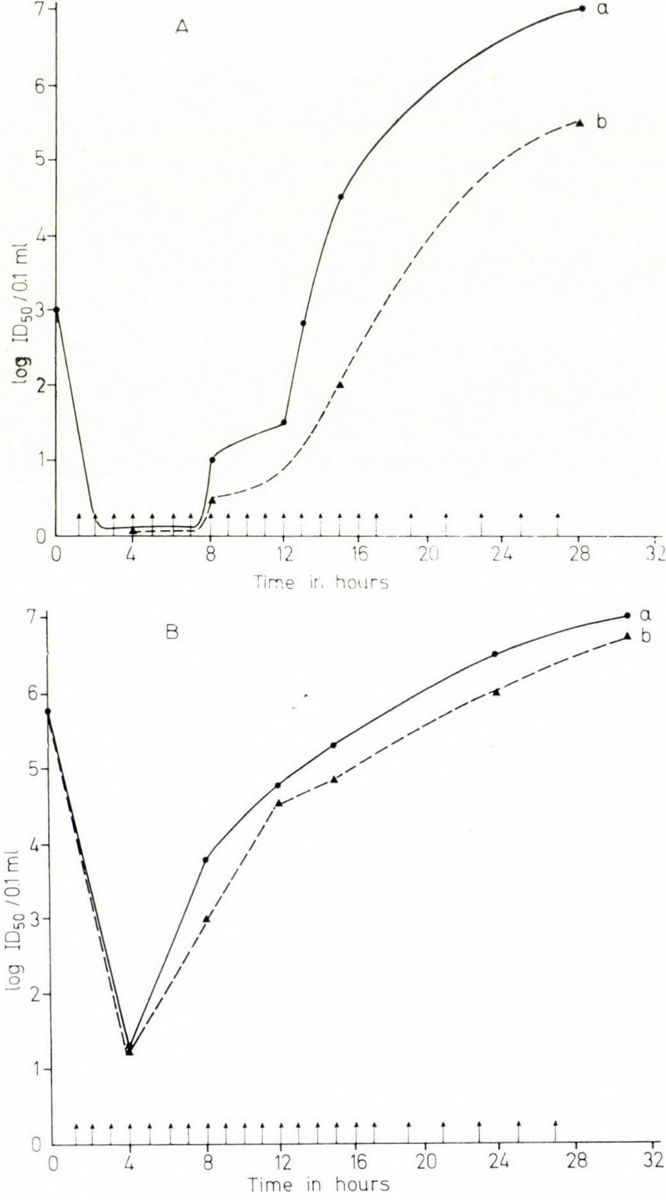

Fig. 2. Inhibition of influenza virus multiplication in the mouse lung by inhalation of ether evaporating from a suspension of *8ac*. Strain: A-1 Budapest 4/49. Inoculum: A: 10^3 ID_{50}, B: 5×10^5 ID_{50}. a = Control; b = *8ac*. Arrows indicate the time of inhalations. For further explanations, see the text

and every second hour from 16 to 27 hours, 21 times altogether. Control mice were placed over peroxide-free ether. For virus titration in the lungs mice were killed at shorter intervals than in Experiments I and II.

The animals tolerated the anaesthesia well. None of those allowed to survive died or showed symptoms of illness during the 9-day period following infection.

The curves in part A of Fig. 2 show the inhibitory effect of the inhaled ether evaporated from the suspension of *8ac*. Inhibition was more pronounced than in Experiment I, in which the inoculum was 1/20th of the inoculum administered in this experiment.

Experiment IV. In this experiment the inoculum was raised to 5×10^5 ID_{50}; other conditions were the same as in Experiment III. Part B of Fig. 2 shows that there was a consistent inhibition even against this large dose of virus. Inhibition was not less pronounced than in Experiment II, although the inoculum was 600 times as large.

Applying the sign test for Experiments III and IV, the 4-hour data were omitted, since by that time the first reproduction cycle had not come to an end. Thus seven values were comparable and the control lungs showed higher titres throughout, $p = 2^{-7} < 1\%$.

For the sake of comparison it may be noted that we have made attempts to achieve an antiviral effect by intraperitoneally administered *8ac* against inocula containing 10^3 ID_{50} or more. However, in these cases an obvious inhibition needed lethal doses of *8ac*. The injected animals died in 48 or 72 hours. If three doses were injected daily, the *pro die* LD_{100} of *8ac* was 150 mg.

Preparation of 8ac-et. The surprisingly high efficiency of the ether evaporated from the *8ac* suspension could not be attributed to the ether itself, as in the control lungs virus multiplication was undisturbed. It was therefore supposed that the active fraction of *8ac* is ether-soluble. To obtain more evidence, we prepared an ether extract from *8ac*. Three g of *8ac* and 100 g freshly distilled ether were shaken for 5 minutes in a balloon flask of 300 ml with glass beads in it. The ether was poured into a centrifuge tube and centrifuged at 3000 r.p.m. The sediment was united with the residuum in the flask. This procedure was repeated four times, each time with 100 ml fresh ether. The supernatants were pooled and shaken with 200 ml saline. Then the ether was evaporated *in vacuo*. The remaining aqueous solution was purple in colour and its surface was covered by a fatty film. The fatty substance was suspended in the aqueous phase by shaking and the suspension was dialyzed against distilled water at 4 °C then the extract, termed *8ac-et*, was lyophilized. The lyophilized extract, weighing 0.30 g, was purple in colour and fatty in consistence.

Experiment V. Extract *8ac-et* was dissolved in saline and its antiviral activity was determined in HORVÁTH's rolling drum against 10^5 ID_{50} of the strain Budapest 4/49. The results, compared to data published in a previous report [4], are shown in Table I. Accordingly, substance *8ac-et* proved to be 4.8 times more active than substance *8ac*.

Table I

Antiviral activity of Francis inhibitor derivatives

Substance	ED_{50} mg/ml	Fiducial limits	Relative efficiency	
K_0	4.64	(2.71—7.57)	1	(—)
8m	0.39	(0.12—1.27)	11.89	(9.9—13.88)
8ac	0.12	(0.04—0.40)	38.66	(32.2—44.3)
8ac-et	0.025	(0.008—0.12)	185.6	(166.8—201.4)

Experiment VI. Mice were infected with 10^4 ID_{50} of virus. In the subsequent 72-hour period each mouse received 12 intraperitoneal injections. Control mice received saline, the others 300 μg of *8ac-et* in 0.5 ml saline each time. Results are illustrated in Fig. 3.

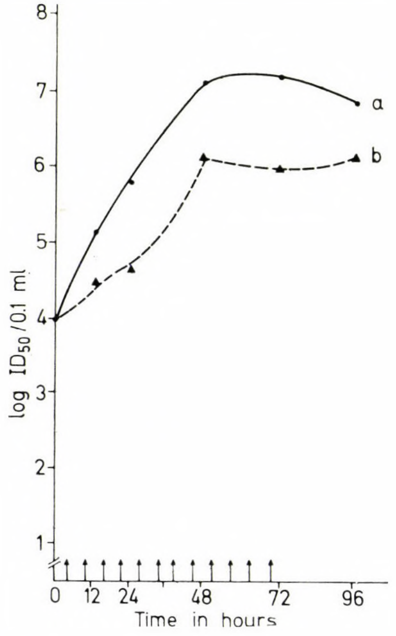

Fig. 3. Inhibition of influenza virus multiplication in the mouse lung. Strain: A-1 Budapest 4/49. Inoculum: 10^4 ID_{50}. a = Control; b = *8ac-et*. Arrows indicate the time of inhalations

Against the large inoculum the inhibition, though consistent, did not exceed 1 log unit. As for the sign test we assumed a unilateral binomial distribution. On the basis of Experiment V we postulated that the virus titre cannot be increased by the aqueous solution of *8ac-et*. According to the sign test, $p = 2^{-5} < 5\%$.

Preliminary data of analytical ultracentrifugation and chemical analysis. Centrifugation at 60,000 r.p.m. resulted in rapid sedimentation of a considerable part of K_0 and *8m*. The fraction that had not settled in 12 minutes formed a well-defined, not quite homogeneous, boundary with $S_{w20^\circ} = 16$ (Fig. 4).

Substance *8ac* proved to be heterodisperse. Fig. 5 shows no distinct boundary. A considerable part of substance *8ac* failed to settle at 50,000 r.p.m. Substance *8ac-et* behaved similarly.

The nitrogen and lipid contents are shown in Table II, the neuraminic acid contents in Table III. Compared to K_0 and *8m*, *8ac*, and especially *8ac-et*, are rich in lipids, first of all in phospholipids and poor in fixed and free neuraminic acid. The component demonstrated as fixed neuraminic acid by the methods applied could not be liberated by digestion with influenza virus. We cannot exclude that the non-digestible "neuraminic acid" was some other component, *e.g.*, malonaldehyde that might have been formed from unsaturated fatty acids. It may be added that in the presence of 2% *8ac* or *8ac-et* the neuramidase activity of the PR8 virus was undisturbed.

Table II

Nitrogen and lipid contents

Substance	Nitrogen per cent	Cholesterol	Total		Reextracted		Total lipid
			lipid	phospholipid expressed in lecithin	lipid	phospholipid expressed in lecithin	
		mg per 100 g					
K_0	6.5	68	1	25	—	—	875
8m	6.5	92	2	50	—	—	500
8ac	1.4	—	53	1325	—	—	3750
8ac-et	8.7	2422	291	7266	241	6016	25380

Table III

Neuraminic acid contents

Substance	Neuraminic acid, per cent		Per cent neuraminic acid liberated from the fixed state by PR8 virus
	free	fixed	
K_0	0.76	3.47	72
8m	0.26	3.13	100
8ac	0.45	0.73	Ø
8ac-et	0.02	1.22	Ø

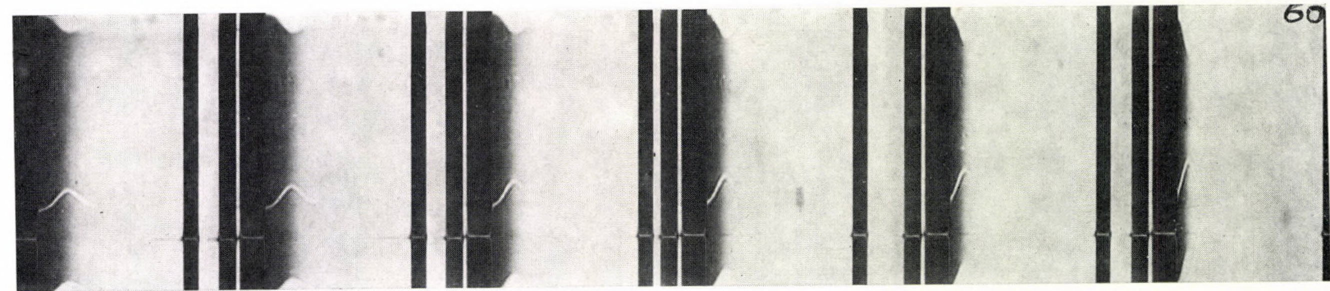

Fig. 4. Analytical ultracentrifugation of substance *8m.* 1% solution, 60,000 r.p.m., 12-minute intervals (from right to left)

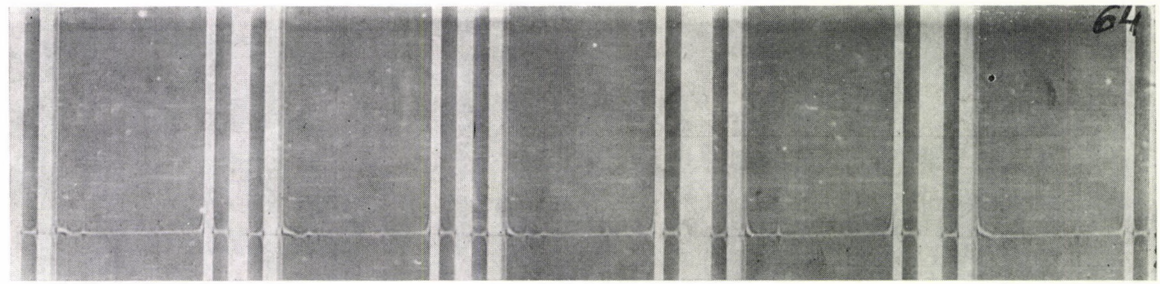

Fig. 5. Analytical ultracentrifugation of *8ac.* 2% solution, 50,000 r.p.m., 9-minute intervals (from right to left)

Discussion

Substance K_0 when administered intraperitoneally failed to inhibit virus multiplication in the mouse lung. *8m* was active against 50 ID_{50}, whereas *8ac* was active even against inocula as large as 10^3 ID_{50}.

In the course of an earlier experiment we made unsuccessful attempts to increase the efficiency of one of our substances by removing lipid contaminants. The substance was subjected to ether extraction. The slightly coloured ether that contained the extracted lipids was subsequently used for anaesthetizing mice. One of our semi-adapted influenza virus strains when passaged in mice anaesthetized in this way was lost in several passages. When the same contaminated ether was distilled *in vacuo*, a coloured distillate was obtained. There is no evidence of the coloured substance being identical with the antiviral agent, yet, it might be supposed that, like the coloured substance, at least part of the antiviral substance forms an azeotropic mixture with ether. Another part of the active substance (*8ac-et*) was recovered in the aqueous extract after the solvent had evaporated.

In previous reports [1, 2] it was demonstrated that the fixation of substance *8m* to the influenza virion was much more stable than the fixation of the Francis inhibitor, the normal receptor analogon of human serum. Thus, we attributed the antiviral activity of *8m* to a competitive inhibition of the adsorption of the virion to the susceptible cell, a phenomenon similar to the nonspecific neutralization of certain strains of influenza A-2 virus by the gamma inhibitor, another α_2-macroglobulin [11, 12], present in the sera of several animal species. Since then it has been shown [13, 14] that the adsorption to and elution from receptor substances are not necessarily related to the neuraminidase activity of the virion. Consequently, the rapid liberation of neuraminic acid from *8m*, presumably due to the alkali treatment during its preparation [15], appears to be compatible with its stable fixation to the virion. Thus, in relation of substance *8m* our concept may be tenable.

On the other hand, the more active substances *8ac* and *8ac-et*, cannot be regarded as receptor analogons. Further investigations are needed to reveal their composition and mechanism of action.

It may be pointed out that the virus strain used in the mouse experiments multiplied in the mouse lung well, but failed to kill the infected mice. The protective effect of substances against the lethal effect of a fully-adapted influenza virus strain is under study. On the evidence of preliminary results, K_0 and *8m* are inactive in this respect.

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INDUCTION AND MULTIPLICATION OF λ-PHAGE

III. THE EFFECT OF HYDROXYUREA ON HEAT INDUCTION

By

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Summary. Hydroxyurea, an anticarcinogenic and DNA-synthesis-inhibiting agent, is similar to mitomycin and ultraviolet irradiation in failing to induce the heat-inducible strain *Escherichia coli* K12 (λcI-857). The effect of hydroxyurea on prophage induction by heat depends on environmental conditions; in complete medium it delays the heat derepression of prophage. In shift-down experiments the process is more sensitive to hydroxyurea.

Hydroxyurea, a recently described anticarcinogenic agent exerts a specific inhibitory action on DNA synthesis [1] and on phage production by infected bacteria [2]. As to inductive phage production, in contrast with the finding of Heinemann and Howard [4] we have shown [3] that hydroxyurea, similarly to mitomycin, induced strain *E. coli* K12 (λ28). The two agents differed, however, in their mode of action. When added together with mitomycin in shift-down experiments, hydroxyurea partly inhibited the induction by mitomycin. Both effects were due to the inhibition of DNA synthesis.

The present paper describes studies on the effect of hydroxyurea on heat-inducible *E. coli* strain K12 (λcI-857), made with the purpose of obtaining new data for the mechanism of induction by heat. This was made possible by the fact that hydroxyurea, similarly to ultraviolet irradiation and mitomycin, does not induce λ-phage production in the test organism at 28 °C.

Materials and methods

Organisms. Heat-inducible strain *E. coli* K12 (λcI-857) *threo*$^-$ *leu*$^-$ was used as lysogenic strain, *E. coli* C600 as indicator strain. The former organism was kindly supplied by Dr. F. Jacob, Institute Pasteur, Paris.

Culture media. Nutrient broth was used as a complete medium. For minimal medium Davis and Mingioli's medium [5] was supplemented with L-leucine and L-threonine (20 μg/ml each). Hydroxyurea was prepared in our institute by S. Sólyom. A molar stock solution was prepared freshly before use.

Inoculum. Complete or minimal medium was inoculated from agar slant cultures and incubated overnight. Then the culture was transferred into 10 volumes of the same medium and incubated for 2 hours. The bacteria were washed twice in distilled water, and inoculated with 5×10^5 cells/ml. The inoculum cultures were grown at 28 °C.

Shift-down experiments. The inoculum culture grown in complete medium was inoculated into minimal medium.

Determination of the number of plaques and infective centres and of phage yield. The plaque count from untreated cultures determined on the indicator strain after incubation at 28 °C was regarded as the number of infective centres. The concentration of complete phages

was estimated by incubating the plates inoculated with chloroform-treated cultures and the indicator strain at 37 °C. Phage yields were determined by diluting 1 : 400 the heat-treated cultures into complete medium at 28 °C, and the number of complete phages was determined after a subsequent incubation for 100 minutes. Other experiments were performed by the standard phage technique [6].

Results

Complete medium inoculated with bacteria grown in complete medium. It is known that heat-inducible λ-lysogenic strains grow well at 28 °C. At this temperature our strain produced no λ-phage spontaneously. If the culture is ex-

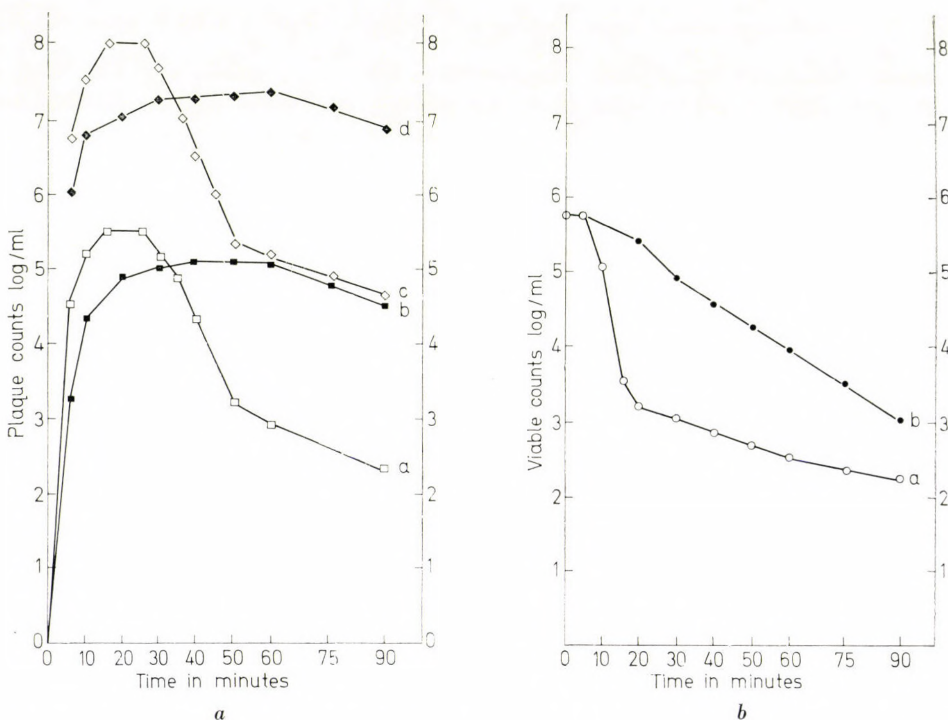


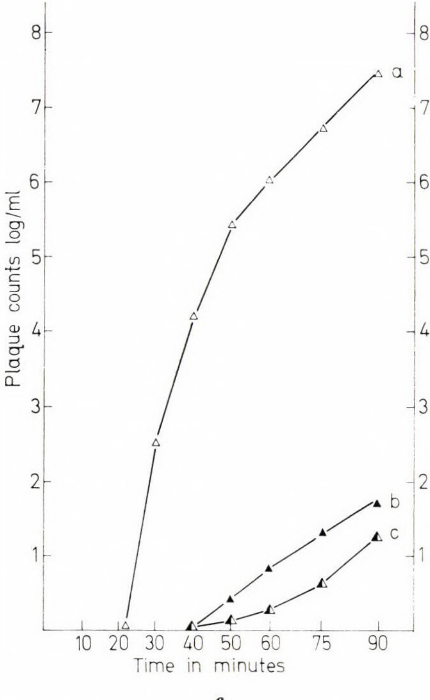
Fig. 1a. Effect of hydroxyurea on heat-induction in complete medium. a = number of infective centres in control culture; b = number of infective centres in the presence of 0.1 *M* hydroxyurea; c = complete phage yield in cultures transferred to 28 °C at different intervals and incubated further for 100 minutes; d = complete phage yield in cultures transferred to 28 °C at different intervals and incubated further for 100 minutes; 0.1 *M* hydroxyurea added at zero time was diluted 1 : 400 when the culture was transferred to 28 °C. In the case of curves c and d, values were multiplied by 400

Fig. 1b. Effect of hydroxyurea on heat induction in complete medium. a = viable cell count in control medium; b = viable cell counts in the presence of 0.1 *M* hydroxyurea

posed to 45 °C, the thermal shock inactivates the immune material stabilizing the lysogenic state (repressor) and the prophage is released. This induction process is complete in the whole population in 15—20 minutes and results in

an increase in the number of infective centres. If the culture is incubated further at 45 °C the vegetative cycle of phage multiplication starts; in a probably late step, however, the process is inhibited and therefore complete phages are produced in small amounts. At the same time the production of protein responsible for lysis is not inhibited and, accordingly, the cells may be lysed before the maturation of the phages [7]; thus the number of infective centres decreases. In contrast, when the culture is cooled after 20 minutes exposure to 28 °C, production of complete phages begins rapidly.

The above process and the effect of hydroxyurea on it are presented in Figs 1a, 1b, and 1c. It is seen that in the presence of 0.1 *M* hydroxyurea the



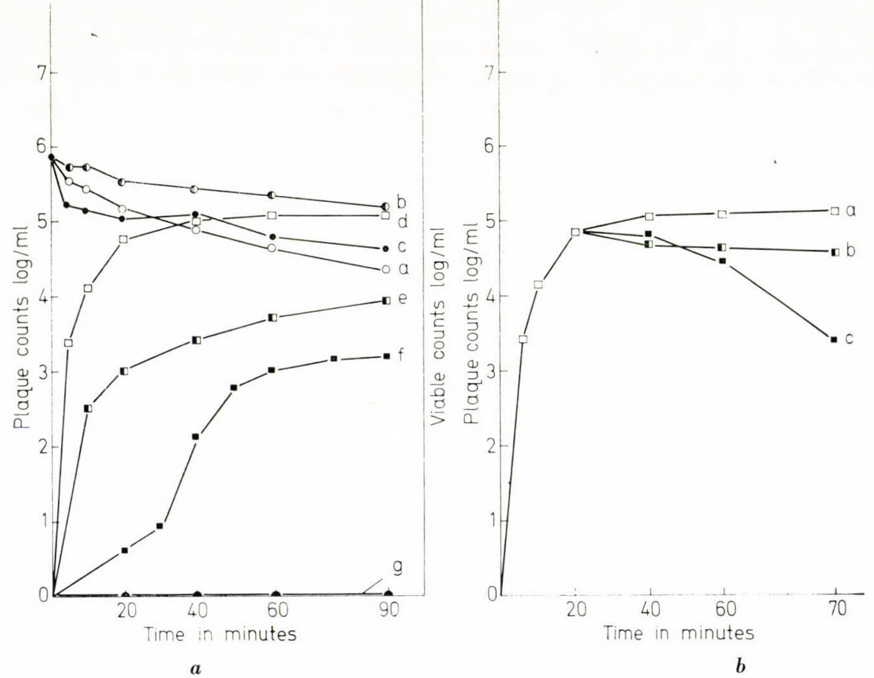
c

Fig. 1c. Effect of hydroxyurea on heat induction in complete medium. a = production of complete phages in cultures transferred to 28 °C after 20 minutes; b = production of complete phages in cultures transferred to 28 °C after 20 minutes in the presence of 0.1 *M* hydroxyurea; c = production of complete phages at 45 °C. All cultures were incubated from zero time at 45 °C

increase in the number of infective centres was less rapid than in the absence of the agent, but the high values reached persisted also at 45 °C (Fig. 1a). While the viable counts decreased rapidly in the control culture, in the presence of hydroxyurea the decrease was less steep (Fig. 1b). If after 20 minutes the culture heated with hydroxyurea was cooled to 28 °C, the production of complete phages was inhibited (Fig. 1c).

To prove that the number of infective centres corresponded to the number of induced cells provided the concentration of complete phages was relatively low, the phage yield was determined at intervals during incubation at low temperature. The phage yield for the number of infective centres at the given interval was approximately 200, independently of the presence or absence of hydroxyurea during heat treatment (Fig. 1a).

The experiments were performed also at 43 °C. The results were similar, except that a considerable amount of complete phages was produced, consequently the number of infective centres showed a rise after an initial decrease. Accordingly, treatment at 43 °C was less suitable for studying lysis-inhibition by hydroxyurea.



a *b*

Figs 2a and *b*. Effect of hydroxyurea on heat induction in shift-down experiment
Fig. 2a. a = viable cell counts in control culture; b = viable cell counts in the presence of 0.01 *M* hydroxyurea; c = viable cell counts in the presence of 0.1 *M* hydroxyurea; d = number of infective centres in the control culture; e = number of infective centres in the presence of 0.01 *M* hydroxyurea; f = number of infective centres in the presence of 0.1 *M* hydroxyurea; g = complete phage yield in culture transferred to 28 °C after 20 minutes

Fig. 2b. a = number of infective centres in control culture; b = number of infective centres in the presence of 0.01 *M* hydroxyurea added after 20 minutes; c = number of infective centres in a culture transferred from 43 °C to 28 °C after 20 minutes. All cultures were incubated from zero time at 43 °C

Shift-down experiments. If bacteria grown in complete medium were inoculated into minimal medium and exposed to 43 °C, the increase in the number of infective centres remained at a high level for long (Fig. 2a). If the cul-

ture was cooled to 28 °C after 20 minutes, the production of complete phages did not begin and even the number of infective centres decreased gradually by approximately 95% (Fig. 2b). The process was reversible; repeated exposure to heat restored the high levels.

If the culture had been kept at 43 °C for 90 minutes then cooled to 28 °C, there was no decrease in the number of infective centres and a small degree of complete phage production was observed.

Heat inductions in shift-down experiments were performed at 43 °C because at 45 °C the hydroxyurea concentration applied was bactericidal; accordingly, the specificity of the effect on phage induction would have been doubtful at 45 °C. The above difference in temperature caused no alteration in the heat induction of control cultures.

The effect of hydroxyurea on heat induction in shift-down experiments can be summarized as follows: 0.1 *M* hydroxyurea was found to delay the increase in number of infective centres; at 0.01 *M* concentration the agent exerted a similar though less definite effect. There was no significant bactericidal effect (Fig. 2a). If 0.01 *M* hydroxyurea was added after 20 minutes exposure to heat, the number of infective centres remained essentially unaltered (Fig. 2b).

Table I

Effect of hydroxyurea at various concentrations on heat induction

Hydroxyurea, *M*	Complete medium				Shift-down	
	No. of infective centres (ml)		No. of viable cells (ml)		No. of complete phages (ml)	No. of infective centres (ml)
	45° 20′	45° 60′	45° 20′	28° 180′	45° → 28°* 150′	43° 20′
—	3×10^5	3×10^2	1.5×10^3	5×10^6	10^8	6×10^4
0.001	2×10^5	3×10^2	2×10^3	4×10^6	8×10^7	9×10^3
0.003	2×10^5	3×10^3	6×10^3	10^6	5×10^6	5×10^3
0.01	2×10^5	5×10^3	5×10^4	9×10^5	2×10^6	10^3
0.3	10^5	1.5×10^4	10^5	6×10^5	8×10^3	8×10^1
0.1	8×10^4	10^5	2×10^5	3×10^5	1.5×10^2	4×10^0

* Temperature-shift after 20 minutes.
All cultures were inoculated with 5×10^5 cells per ml grown in complete medium.

Table I summarizes the effect of different hydroxyurea concentrations on the number of infective centres, number of surviving bacteria, production of complete phages in complete medium and the number of infective centres in shift-down experiments. It is seen that a bacteriostatic concentration was needed for inducing any of the above effects.

Minimal medium without shift-down. If bacteria grown in minimal medium were transferred into minimal medium, the rise in the number of infective centres and the decrease in viable cell counts were similar to results obtained in complete medium. The retarding effect of hydroxyurea on the rise of infective centres' titre was stronger than in complete medium but weaker than in shift-down experiments (Fig. 3).

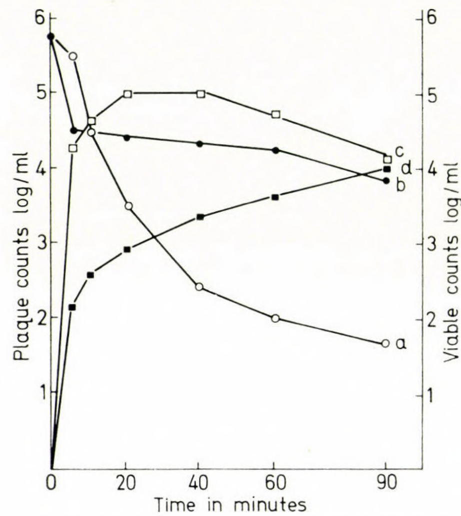


Fig. 3. Effect of hyroxyurea on heat induction in minimal medium without shift-down. a = viable cell counts in control culture; b = viable cell counts in the presence of 0.1 *M* hydroxyurea; c = number of infective centres in control culture; d = number of infective centres in the presence of 0.1 *M* hydroxyurea. All cultures were incubated from zero time at 43 °C

Discussion

In these experiments the effect of hydroxyurea on the production of heat-inducible λ-phage was studied in detail. Some conclusions could be drawn as to the mechanism of induction by heat.

Hydroxyurea is known to be a specific inhibitor of DNA synthesis [1]. It would appear that hydroxyurea inhibits only the replication of phage DNA. This would explain not only the inhibition of complete phage production in induced cultures transferred to 28 °C but also the phenomenon that the number of infective centres does not decrease in complete medium containing hydroxyurea after 25 minutes at 45 °C. As mentioned above, the fall in the number of infective centres in the control culture is due to the prematuration lysis described by GROMAN and SUZUKI [7]. A significant lysis occurs only when there is a phage-DNA synthesis [8].

We have observed that, especially in shift-down, hydroxyurea delays the increase in the number of induced cells. As in heat induction the immune

substance responsible for the lysogenic state is inactivated, *i.e.*, prophage repression ceases, the experiments indicate that hydroxyurea interferes with heat-induced derepression. Accordingly, in addition to inhibiting phage-DNA replication, hydroxyurea should have another site of action.

This hypothesis is disputable if we suppose that the inhibition by hydroxyurea is virtual: the immune material is inactivated in the presence of hydroxyurea, but the majority of cells produces no plaques because during the latency period needed for the starting of phage-DNA synthesis the immune substance is reproduced or reactivated.

This objection can be excluded on the basis of the following assumption. Provided that the replication of phage-DNA due to heat induction in the shift-down condition did not start till the 20th minute, the addition of hydroxyurea ought to cause a definite decrease in the number of infective centres during a subsequent incubation for 20 minutes. After the dilution of hydroxyurea the beginnings of DNA synthesis would require a latency period also in this case and thus there would be a possibility to restore the immune state. According to the experimental results, however, the addition of hydroxyurea exerts no effect at all. In view of this it remains to be proved that phage synthesis does not begin till the 20th minute. It was shown that cooling of the culture at that time causes a 95% decrease in the number of infective centres. The decrease being reversible, it must be the consequence of the restoration of the immune state. After the beginning of phage-DNA replication the immune material cannot inhibit phage maturation and the number of infective centres could not be decreased at low temperature.

Hypotheses for heat induction can be divided into two groups. According to the assumption accepted by the majority of authors the repressor is inactivated directly by heat treatment [9—11]. Other investigators suppose an indirect mechanism to be involved. According to Sussman and Jacob [12] and Wainfan *et al.* [13], production of the repressor is inhibited. In the opinion of Goldthwait and Jacob [14] in mutant T44 an adenine derivative accumulating as a result of heat-treatment inactivates the repressor.

The present observation that hydroxyurea inhibits also derepression cannot be explained by a direct heat-inactivation. The finding indicates the role of an indirect mechanism associated with DNA metabolism.

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BREAKDOWN OF OLIGOSACCHARIDES BY SOME AFERMENTATIVE YEAST SPECIES

By

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Summary. The breakdown of maltose, sucrose and raffinose by living and acetone-treated cells and cell-free extracts of four afermentative yeasts has been examined. *Bullera tsugae, Sporobolomyces singularis, Debaryomyces vini* and *Rhodotorula zsoltii* showed uniformly an acetone-sensitive maltose breakdown. Enzymes similar to those playing part in this process occur in several other yeasts. The identity of the extracellular sucrose-decomposing enzyme of *R. zsoltii* with invertase is doubtful.

The taxonomic importance of oligosaccharide breakdown by yeasts is well known. It is clear that in addition to examining the utilization reactions of various species, the mechanisms involved in these processes should also be studied [1, 7, 8]. We have pointed out that in this respect the first steps of oligosaccharide metabolism (transport and cleavage) are important, since the metabolism of the subsequently produced monosaccharides is uniform [2—5, 7, 12]. In this paper we present further experiments on the enzymatic basis of the utilization of diagnostic oligosaccharides.

Materials and methods

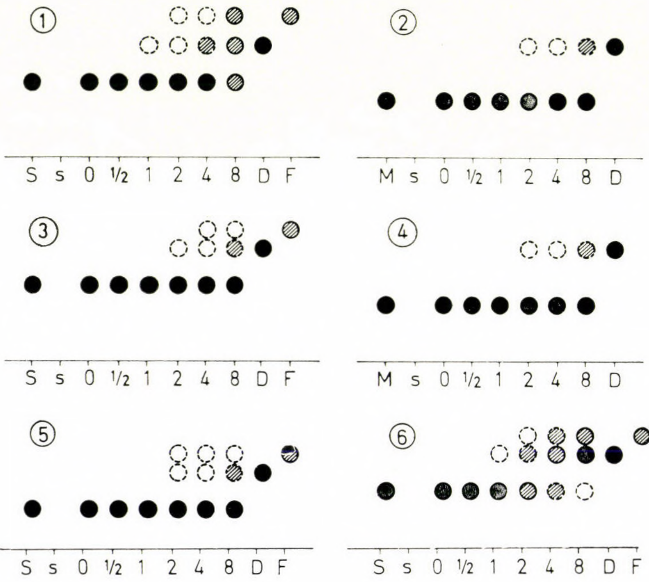
The following *strains* were used. (1) *Bullera tsugae* Phaff strain 60—71; assimilating glucose, sucrose and maltose, weakly assimilating lactose. (2) *Debaryomyces vini* our strain 65/XLI; assimilating glucose, sucrose and maltose. (3) *Rhodotorula zsoltii* our strain 64/XLV; assimilating glucose, weakly assimilating galactose and sucrose. (4) *Sporobolomyces singularis* Phaff strain 60—79; assimilating glucose and lactose.

The *methods* were similar to those described in our previous papers. The yeasts were grown on Csillag's molasses agar. The harvested cells were washed three times in saline and divided into three parts. (1) For the examination of living cells the centrifuged deposit was resuspended in $M/15$ pH 7.2 phosphate buffer so that the suspension contained 0.5 g wet weight cells per ml (usually 1.25 ml buffer for 1 g wet cells). Then to 1.5 ml suspension 1.5 ml of 4% sucrose, maltose or raffinose solution was added. The mixtures were incubated in small test tubes. Samples were taken at 0, 0.5, 1, 2, 4 and 8 hour intervals. Enzyme activity was terminated by 2 minutes boiling then 2 μl aliquots were chromatographed as described in reference [1]. (2) Acetonized cells were prepared by resuspending then washing the deposit twice in 2 volumes of acetone at 4 °C. After drying the cells at room temperature a suspension was prepared with the above-mentioned phosphate buffer (representing 0.5 g wet weight cells per ml). Further experiments were carried out as described above. (3) Cell-free extracts were prepared by grinding the deposit with quartz sand in a chilled mortar for 10 minutes. The ground material was washed into a centrifuge tube so that to 1 g of the original wet cells 1.25 ml of the above phosphate buffer was added. The material was centrifuged at 2500 *g*, then the supernatant was centrifuged again at 3000 *g*. The used proportion of phosphate buffer and

cells (1.25 : 1) was fixed for the living suspension; for the other two preparations we attempted to approximate the same cell contents. In preparing the extract the needed amount of material was calculated by assuming a complete disintegration and extraction; this was indicated by a strong opalescence and high protein content (usually 2.0—2.5 mg/ml) of the extract. Further experiments were performed as described above.

Results and discussion

Under semi-anaerobic incubation conditions living cells of *Bullera tsugae* caused neither a decrease in the amount of the examined three sugars nor the appearance of breakdown products. Similar observations were made with



Figs 1 and 2. Sucrose and maltose breakdown by cell-free extract of *Bullera tsugae.* The system contained in a final volume of 3 ml *M*/30, pH 7.2 phosphate buffer extract representing 750 mg wet weight cells and 60 mg sugar. Left-hand side of chromatograms: sucrose, maltose and extract controls; right-hand side of chromatograms: glucose (D) and fructose, or glucose (D) controls. Figures under the start line show sampling intervals in hours

Figs 3 and 4. Sucrose and maltose breakdown by cell-free extract of *Sporobolomyces singularis.* For explanation, see Figs 1 and 2

Figs 5 and 6. Sucrose breakdown by living cells and cell-free extract of *Rhodotorula zsoltii.* The system contained in a final volume of 3 ml *M*/30, pH 7.2 phosphate buffer 750 mg living cells (Fig. 5) or extract representing the same amount of wet weight cells (Fig. 6) and 60 mg sugar. Left-hand side of chromatograms: sucrose and suspension or extract controls; right-hand side of chromatograms: glucose (D) and fructose controls. For further explanations see Figs 1 and 2

acetone-treated cells. In contrast the cell-free extract cleaved sucrose and maltose to monosaccharides but exerted no effect on raffinose (Figs 1 and 2).

From the results it has been concluded that, (1) the cells produce neither invertase (raffinose is not attacked and sucrose is decomposed only by the

cell-free extract) nor melibiase (raffinose is not attacked); (2) the culture produces maltose- and sucrose-cleaving enzymes (α-glucosidase sensitive to acetone and localized intracellularly); these sugars are not attacked by acetonized and living cells.

Sporobolomyces singularis yielded qualitatively similar results as *B. tsugae* but was somewhat less active (Figs 3 and 4). As the former species fails to assimilate sucrose and maltose it is evident that it contains no transport systems specific for the two disaccharides. The presence of intracellular sugar-cleaving enzyme(s), in view of the lack of transport system(s), indicates that *Sp. singularis* is derived from a similar, but sucrose and maltose-assimilating species, perhaps from *B. tsugae* which produces also acetone-sensitive intracellular enzymes acting upon maltose and sucrose. This hypothesis is supported by the fact that in failing to produce pigment *B. tsugae* resembles *Sp. singularis*, which is the first colourless species described in the genus *Sporobolomyces* and also by the observation that, unlike other yeasts, *B. tsugae* and *Sp. singularis* exert a special effect on lactose (this property, in view of the inductive nature of the enzyme, is not dealt with in this report).

Debaryomyces vini was identical in sugar decomposition with *Sp. singularis*. However, as this species assimilates both sucrose and maltose, the breakdown of these sugars could be expected.

Living cells of *Rhodotorula zsoltii* exerted a very weak extracellular sucrose breakdown and did not attack maltose and raffinose. Acetonized cells were inactive against all three sugars. The cell-free extract cleaved sucrose fairly, raffinose weakly and was ineffective in maltose (Figs 5 and 6). The results indicate the presence of an invertase-like enzyme (extracellular activity against sucrose, raffinose breakdown into melibiose and fructose), which is weakly active or is produced in small amounts. The inactivity of acetonized cells seems to speak against this consideration. Our previous experiments showed that acetonized cells of invertase-producing yeasts decomposed both sucrose and raffinose [1, 6]. These preparations may have displayed a partial acetone sensitivity which, however, remained undetected with the semiquantitative method used. It may be assumed that in the present study this weak activity, after a possible inactivation by acetone, decreased below the detectable level even for sucrose breakdown while an initially weaker activity was responsible for the doubtful breakdown of raffinose. The fact that a subminimal release of fructose is not sufficient for growth in raffinose (negative assimilation test) is probably also due to the low "invertase" level and the decreased activity on raffinose. Similar observations were made on *Rhodotorula slooffii* [9] and important differences were observed between industrial yeasts as to the production and release of invertase [11]. On the other hand, invertases of various yeast species differed in the decomposition of transfer oligosaccharides produced by *Claviceps* invertase [10].

As a general consideration it should again be stressed that in our semi-anaerobic incubation system (relatively high amounts of medium in narrow tubes incubated without shaking) living cells of the examined afermentative species did not attack the oligosaccharides. Although the four strains uniformly failed to produce extracellular enzymes, the cell-free extract of three of them exerted a good sugar-decomposing activity. In view of the afermentative character of the examined species it may be concluded that the lack of an anaerobic transport activity is responsible for the incapability of the cells to attack the intracellularly cleavable sugars. An alcohol-dehydrogenase deficiency cannot be supposed to occur in many species, since the ethanol assimilation observed in these organisms is started by that enzyme.

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EFFECT OF ENDOTOXIN ON THE SERUM LEVEL OF COMPLEMENT COMPONENTS

II. EFFECT OF ENDOTOXIN ON DOG SERUM COMPLEMENT LEVEL IN VIVO AND IN VITRO

By

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Summary. The effect of *Serratia marcescens* endotoxin on the level of complement and its components has been examined in the dog. The role of "natural" antibodies in the endotoxin-induced decrease of the complement level has also been studied.

Intravenous injection of sublethal endotoxin dose or incubation of dog serum with endotoxin *in vitro* caused a significant decrease in the level of total complement and its four "classical" components.

No correlation was revealed between "natural" antiendotoxin titre and endotoxin-induced decrease in complement activity in individual human sera or in pooled sera from various animals; the decrease was significant after the absorption of antiendotoxin.

The results and other authors' data point to a probable part of the endotoxin-activated complement system in the mechanism of endotoxin shock. The endotoxin-antiendotoxin reaction does not seem to be associated with, or is only partly responsible for, the decrease in complement level.

Intravenous injection of bacterial endotoxin causes a change in the serum complement level. Large doses decrease it in 10 to 80 minutes. After a few hours the titre rises to the normal value and after 24 to 48 hours it exceeds the original level (rebound effect [1—3]). If small doses are given the complement level reaches a maximum in 48 hours without any previous decrease [4]. Of the four classical complement components C′2 and C′4 increase significantly, but the other two components remain unaltered. This finding is probably explained by a direct stimulating effect of endotoxin on serum protein synthesis [5]. In the present study the behaviour of complement components has been investigated in dogs injected intravenously with sublethal doses of endotoxin. This seemed to be interesting since the decrease in total complement level did not yet prove the role of the complement system in the mechanism of endotoxin shock. We expected to draw more definite conclusions from the behaviour of the components, especially of C′3.

In subsequent experiments the role of "natural" antibodies in endotoxin-induced changes of the complement level was examined. Several authors have emphasized the influence of antigen-antibody reaction on this phenomenon, but many others claimed to have observed no such association. In our experiments we studied the effect of antiendotoxin detectable with haemagglutination and the behaviour of sera after the absorption of these antibodies.

Materials and methods

Sera. Human, guinea pig, dog, rabbit and rat sera were used. Unless otherwise indicated, pooled sera from 3 to 8 animals were prepared.

Endotoxin. Serratia marcescens endotoxin was prepared in two different manners: (i) Boivin-Mesrobeanu extract kindly supplied by Dr. K. Ujhelyi of our institute; (ii) endotoxin prepared as described by Shear and Turner [6].

Complement reagents. R reagents for determining the four classical complement components were prepared from guinea pig serum as described by Kabat and Mayer [7]. The reagents were standardized by the use of freeze-dried guinea pig reference serum.

Dilutions were made with pH 7.4 veronal buffer.

Animal experiments. The endotoxin was injected into, and the blood samples were taken from, the femoral vein under hexobarbital anaesthesia.

Determination of the level of complement and its four components was performed as described previously [4] except that incubation with sensitized erythrocytes lasted for 60 minutes (optimal for dog complement).

Determination of the natural antibody titre was carried out by haemagglutination test using sheep erythrocytes sensitized with endotoxin as described by Skarnes [8]. The test was performed with inactivated sera in Takátsy's microtitrator [9]. The antibody titre was expressed as the reciprocal of the highest dilution giving complete haemagglutination. All sera were checked with normal sheep erythrocytes.

Antibody absorption was performed by the method of Skarnes [8] with endotoxin sensitized sheep erythrocytes.

Results

I. Experiments in vivo. A total of 11 dogs weighing 8 to 11 kg was used First the control experiments were performed on each dog then, after one week interval, the endotoxin was injected.

1. Pyrogen-free saline corresponding in volume to the endotoxin dose was injected intravenously to each of 6 normal, untreated dogs. Blood samples of 3 to 4 ml were taken before and 10, 30, 60, 90 and 120 minutes after injection. The specimens were left to stand at room temperature for 30 minutes then the clot was separated from the tube wall. After another 30 minutes standing at room temperature the specimens were refrigerated for 30 minutes then centrifuged. The sera were stored in the deep-freezer (—16 to —18 °C). The complement level was determined on the next day. Table I presents the titres in 50% haemolytic units ($C'H_{50}$) for complement and its components in the control and 60 minute specimens.

Table I

Effect of saline injection and bleeding on the level of complement and complement components in dogs

	T		C'1		C'2		C'3		C'4	
	$C'H_{50}$	%	$C'H_{50}$	%	$C'H_{50}$	%	$C'H_{50}$	%	$C'H_{50}$	%
Initial value	54	100	315	100	110	100	111	100	360	100
60 min value	59	109	337	107	133	111	109	98	433	120
Difference	+5	+9	+22	+7	+23*	+11	—2	—2	+73*	+20

* Significant difference ($p < 0.05$).

It is seen that the titres for total complement, C′1 and C′3 remained unaltered, those for C′2 and C′4 increased significantly.

This finding may be explained by the fact that the sympathetic stimulation exerted by blood sampling caused the level of these components to increase.

2. 1.8 mg/kg *S. marcescens* endotoxin was given to 11 dogs intravenously. Blood specimens were taken as in the control experiments. In 8 out of 11 dogs

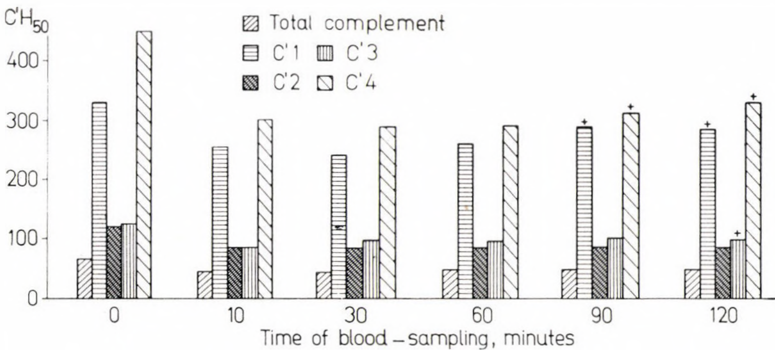

Fig. 1. Effect of endotoxin on the level of complement and its components. Experiments in dogs *in vivo*

the complement titre decreased. In the remaining 3 dogs no alteration was demonstrated between 10 and 120 minutes after injection. Fig. 1 shows data for the 8 dogs with decreased complement level.

The total complement and component levels decreased considerably as early as 10 minutes after injection. Subsequently the total complement and C′3 showed a moderate rise. The decrease in C′1 and C′4 was the most marked after 30 minutes, that in C′2 after 60 minutes. The difference between the initial titre and levels after endotoxin injection was significant for total complement and all its components in the 10, 30 and 60 minute samples. In the 90 minute sample only the total complement, C′2 and C′3, in the 120 minute sample only total complement and C′2 showed significant differences as compared to the original levels. Significance was tested by the *t* method (Fig. 1).

3. At the beginning of the experiments it was already observed that, in contrast to control samples showing normal clotting, blood specimens taken after endotoxin injection failed to coagulate completely; in some of them definite thrombolysis was revealed after separation of the clot from the tube wall. In the deep-frozen serum samples pedunculate precipitation appeared after thawing. Recalcification time of citrated plasma was determined in 6 dogs (Table II).

It was shown that as opposed to a 4 minute average in the control, 10 minute samples showed 9 to 120, 60 minute samples $4^1/_2$ to 25 minute values.

Table II

Effect of intravenous injection of S. marcescens endotoxin on the recalcification time of citrated plasma

No. of dog	Time of blood sampling, minutes			
	0	10	60	120
2	5′	120′	25′	8′
3	4′	25′	4′	1′30″
4	3′	9′	4′30″	2′20″
10	4′30″	18′	8′	2′30″
14	3′10″	40′	4′15″	1′45″
15	4′	60′	60′	5′45″

Two hour samples, with two exceptions, were characterized by recalcification times shorter than those in the controls.

The above phenomenon occurred in all 8 samples showing decreased complement titres after the injection of endotoxin. Coagulation was normal in 3 dogs in which the component remained at the original level after endotoxin injection. In two dogs dying one day after endotoxin injection severe haemorrhagic changes were revealed in several organs.

II. Experiments in vitro. 1. In vitro alterations in complement component levels due to endotoxin were examined as follows. One ml aliquots of dog serum were incubated with 0.2 ml aliquots of endotoxin dilutions at 37 °C for 60 minutes, then the component levels were determined. The control serum was incubated similarly but without endotoxin. Table III shows changes induced by endotoxin diluted 1 : 50 (72 μg/ml).

The level of complement and of the four components decreased significantly. Larger or smaller endotoxin doses caused similar alterations but the decrease of individual components varied in proportion.

Table III

Effect of endotoxin on the level of complement and its components in vitro

	T		C′1		C′2		C′3		C′4	
	$C'H_{50}$	%	$C'H_{50}$	%	$C'H_{50}$	%	$C'H_{50}$	%	$C'H_{50}$	%
Control	60	100	337	100	116	100	98	100	365	100
72 μg endotoxin per ml serum . .	19	32	231	69	63	54	60	61	194	53
$C'H_{50}$ fixed	41*	68	106*	31	53*	46	38*	39	171*	47

* Highly significant difference ($p < 0.01$).

2. The kinetics of the reaction were examined by incubating 1 ml aliquots of dog serum with 72 μg endotoxin in a water bath of 37 °C; the control samples were incubated in a similar manner without endotoxin. Titrations were performed in aliquots incubated for various intervals. Fig. 2 indicates the time course of the reaction expressed as the difference in complement level between the test and control serum, that is, the number of fixed $C'H_{50}$.

After 5 minutes incubation more than 50% of $C'H_{50}$ were fixed. After 60 minutes practically the total complement activity was lost and no further change occurred during incubation for 4 hours.

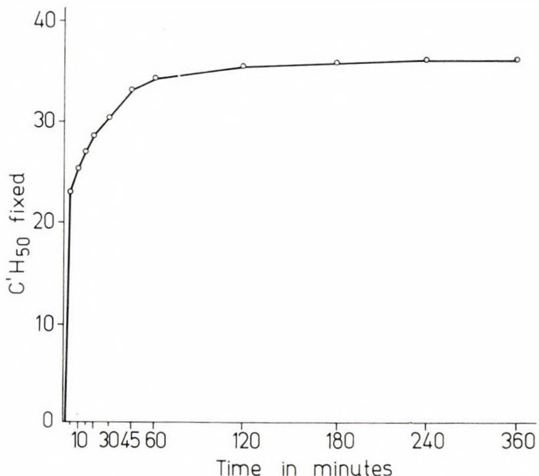

Fig. 2. Time course of endotoxin-complement reaction

3. In subsequent experiments we examined the fixation of complement by 72 μg endotoxin in one hour incubation at various temperatures (Table IV).

The number of fixed $C'H_{50}$ decreased parallel with the lowering of the temperature; however, complement fixation still occurred at 0 °C.

As this finding is in disagreement with the observation of BLADEN *et al.* [10] who were unable to show a decrease in complement titre in guinea pig serum after 18 hours incubation at 0 °C, we performed parallel experiments with guinea pig and dog serum. Table V shows the level of total complement and components in the two kinds of serum after incubation at 0 °C for 60 minutes.

From Table V it is evident that 72 μg endotoxin per ml caused a 27% decrease in $C'H_{50}$ in the dog serum but failed to exert any effect in the guinea pig serum. The two low-titre components C'2 and C'3 remained unchanged in the guinea pig serum; in dog serum each of them decreased by 25%.

Table IV

Effect of incubation temperature on endotoxin-complement reaction

Incubation temperature °C	Complement titre in control*	Complement fixed by endotoxin**	
		$C'H_{50}$	%
37	53.1	35.1	66
30	53.9	34.3	64
22	54.4	30.4	56
16	55.9	24.0	43
10	55.9	19.8	35
0	56.0	17.6	31

* Incubation lasted for 60 minutes at the indicated temperature.
** 72 μg *S. marcescens* endotoxin per ml serum, 60 minutes incubation.

Table V

Effect of endotoxin on the level of complement and its components in dog and guinea pig serum incubated at 0 °C

	Complement and components fixed by endotoxin*									
	T		C'1		C'2		C'3		C'4	
	$C'H_{50}$	%	$C'H_{50}$	%	$C'H_{50}$	%	$C'H_{50}$	%	$C'H_{50}$	%
Dog serum**	16.5	27	46	16	40	25	24	25	66	27
Guinea pig serum***	29.0	8	72	6	0	0	13	3	0	0

* 72 μg *S. marcescens* endotoxin per ml serum, 60 minutes, 0 °C
** Initial $C'H_{50}$: T = 61.5, C'1 = 282, C'2 = 120, C'3 = 96, C'4 = 399
*** Initial $C'H_{50}$: T = 366, C'1 = 1170, C'2 = 750, C'3 = 422, C'4 = 16310

III. Studies on the role of "natural" antibodies. 1. The influence of natural antibodies in human sera on the endotoxin-induced decrease in the complement level is shown in Table VI.

It is seen that there is no correlation between the natural antibody titre and the complement level after endotoxin treatment. The degree of complement fixation may be similar in sera with high and with low antiendotoxin titres, but may be very different in sera showing the same antiendotoxin titre. The correlation coefficient between antibody titre and the number of fixed $C'H_{50}$ was 0.18 (not significant, $p > 0.05$).

In subsequent experiments pooled sera from various animal species were tested. Similarly as in human sera, there was no association between the titre of natural antibodies and endotoxin-induced decrease in complement.

Table VI

Anti-endotoxin titre and endotoxin-induced decrease in complement level in individual human sera

Specimen	Titre of haemagglutination	Initial $C'H_{50}$	Complement fixed*	
			$C'H_{50}$	%
1	3	70	49	70
2	3	69	32	46
3	3	76	17	22
4	4	58	19	33
5	4	100	31	31
6	8	78	35	45
7	12	75	41	55
8	16	91	28	31
9	16	75	40	53
10	16	84	55	67
11	16	68	35	51
12	16	95	27	28
13	16	68	28	41
14	24	87	28	32
15	24	74	41	56
16	32	80	42	53

* 72 μg *S. marcescens* endotoxin per ml serum, 60 minutes, 37 °C

The highest amount of complement activity was fixed in rat serum containing no detectable amount of natural antibodies against *S. marcescens* endotoxin. The highest absolute amount of $C'H_{50}$ was fixed in guinea pig serum with a low antibody titre (1 : 2). In human and dog sera containing antibodies in relatively higher titres, a lower degree of decrease in complement activity was observed (Table VII).

In subsequent experiments the effect of two endotoxin preparations was compared (Table VIII).

The examined sera showed identical antibody titres for the two kinds of preparations. The complement level-decreasing effect of Boivin extract was much more marked than that of Shear extract.

2. Absorption of natural antibodies from dog serum with endotoxin-sensitized erythrocytes gave uniform results in four different experiments. The results are summarized in Table IX.

Although natural antibodies had been completely removed, the decrease in complement level after the addition of endotoxin was only little smaller

Table VII

Anti-endotoxin titre and endotoxin-induced decrease in complement level in pooled human and animal sera

Serum	Titre of haemagglutination	Initial $C'H_{50}$	Complement fixed*	
			$C'H_{50}$	%
Human	16	69	28	41
Dog	16	53	12	23
Guinea pig	2	339	161	47
Rat	0	61	52	76
Rabbit	2	10	0	0

* 36 μg *S. marcescens* endotoxin per ml serum, 60 minutes, 37 °C.

Table VIII

Anti-endotoxin titre and decrease of complement activity by two different endotoxin preparations

Serum	Initial $C'H_{50}$	Boivin-Mesrobeanu extract*			Shear extract*		
		Titre of haemagglutination	Complement fixed		Titre of haemagglutination	Complement fixed	
			$C'H_{50}$	%		$C'H_{50}$	%
Human	51	8	37	73	12	15	31
Dog	45	2	35	78	2	9	20
Guinea pig	402	6	235	59	8	51	13

* 200 μg endotoxin per ml serum, 60 minutes, 37 °C.

Table IX

Effect of antibody absorption on endotoxin-induced decrease in complement level

Dog serum	Titre of haemagglutination	Initial $C'H_{50}$	Complement fixed*	
			$C'H_{50}$	%
Unabsorbed	16	50.5	27.1	54
Absorbed**	0	30.4	14.4	47

* 72 μg *S. marcescens* endotoxin per ml serum, 60 minutes, 37 °C.
** Absorbed twice at 10 °C with endotoxin-sensitized sheep erythrocytes.

than in the unabsorbed control serum. The initial complement titre in the absorbed serum was considerably lower than in the unabsorbed serum. We have no explanation to offer for this finding; on the basis of other experiments we suppose that the decrease was due to a reaction between erythrocytes and natural anti-erythrocyte antibodies.

The effect of endotoxin on the level of complement components in unabsorbed and absorbed serum is presented in Table X.

Table X

Effect of endotoxin on the level of complement components in unabsorbed and absorbed dog serum

Components	Unabsorbed dog serum*			Dog serum absorbed with endotoxin-sensitized erythrocytes**		
	Initial $C'H_{50}$	Components fixed***		Initial $C'H_{50}$	Components fixed***	
		$C'H_{50}$	%		$C'H_{50}$	%
C′1	292	186	63	168	108	64
C′2	72.0	50.2	70	39.3	29.4	75
C′3	75.0	26.4	35	60.0	15.9	27
C′4	307	127	41	257	161	37

* Haemagglutination titre: 16.
** Haemagglutination titre: 0.
*** 72 μg *S. marcescens* endotoxin per ml serum, 60 minutes, 37 °C.

It is evident that there is no significant difference between the two sera in the proportion of fixed component units.

Discussion

After the basic observation of Spink and Vick [1], many authors [2, 3, 10—12] have shown that endotoxins decrease the total complement level both *in vivo* and *in vitro*. From these findings limited conclusions can only be drawn as to the role of the complement system in the mechanism of endotoxin shock. The decrease in the complement level *per se* does not yet prove the activation and role of the complement system in the pathological process since the decrease may be a result of individual changes in the amount of one or more complement components. Accordingly, determination of total complement level should be supplemented with the examination of each component. The endotoxin-induced alteration in complement components was examined by Bladen *et al.* [10] and Gewurz *et al.* [11] in guinea pig serum *in vitro* and by Miler *et al.* [12] in new-born precolostric pigs.

We have shown that the intravenous injection of endotoxin as well as the addition of endotoxin to the serum *in vitro* decreased the level of the four classical components. This finding indicates that the endotoxin activates the whole complement system including the classical component C′3 which is the last in sequence. The extensive studies of Osler *et al.* [13] have proved that

in the course of the activation of the complement system by antigen-antibody complexes anaphylatoxin is produced parallel with the decrease in the C′3 level.

Recent studies have indicated that the classical C′3 component contains several subcomponents (C′3, C′5, C′6, C′7, C′8 and C′9). In the course of the activation of the complement system anaphylatoxins are produced from C′3 and C′5 [14—16]; the reaction needs the presence of components acting earlier in the sequence (C′1, C′4, C′2).

It is known that anaphylatoxin may liberate considerable amounts of histamine. SPINK *et al.* [17] showed that intravenous endotoxin caused in the dog an increase in histamine concentration parallel with the decrease in complement level.

If the above data and our results are compared it seems probable that one site of the endotoxin action is the complement system and that biologically active substances produced from the activated complement components (anaphylatoxins and other unknown factors) play a part in the process of endotoxin shock. This hypothesis has been supported by the finding of GEWURZ *et al.* [11] who showed that, as an effect of chemical detoxication by different procedures, the mouse-toxicity and complement-fixing capacity of endotoxin are undergoing a decrease.

In blood specimens of dogs showing decreased complement levels after intravenous endotoxin injection, coagulation disorders such as absence of clotting, delayed coagulation, prolongation of recalcification time and in some cases thrombolysis have been demonstrated. In deep-frozen plasma specimens a pedunculate precipitate was observed after thawing. In the blood of dogs showing no decrease in the complement level after endotoxin injection no coagulation disorders were observed. GANS *et al.* [18] showed in the dog a prolongation of recalcification time 15 minutes after endotoxin injection. LILLEHEI and MACLEAN [19] found a prolonged coagulation time $1^1/_2$ hours after the injection of lethal endotoxin dose. GANS *et al.* also demonstrated that after intravenous endotoxin injection the plasminogen level decreased and the activity of plasminogen-activator increased, that is, the plasminogen-plasmin system was mobilized.

In contrast to the above findings in dogs, KLEINMAIER *et al.* [20] showed increased coagulability and intravascular thrombus formation after endotoxin injection in the rabbit. The difference between the two species is explained by the fact that in rabbits the plasminogen-plasmin system is not activated by endotoxin [21].

Part of our results (thrombolysis, delayed coagulation) can well be explained by the effect of the activated plasminogen-plasmin system. It has long been known that plasmin activates the complement system *in vitro* [22, 23]. It may, therefore, be supposed that the plasminogen-plasmin system is partly

responsible for the endotoxin-induced activation of the complement system. To confirm this hypothesis, further experiments are needed.

Our results obtained with dog serum *in vitro* differ from those of the authors using guinea pig serum [10, 11].

1. During incubation at 37 °C, in the guinea pig serum only the C′3 level decreases, the remaining three classical components show unaltered titres. In contrast, in dog serum all four components decrease significantly. The cause of the difference is not known; qualitative and quantitative differences in the complement components as well as in the methods used may be involved [24].

2. During incubation at 0 °C the complement level in guinea pig serum shows no decrease while in dog serum the decrease is significant. In our experiments the difference was due to a different fixation of two components (C′2 and C′3) in the two kinds of serum.

In subsequent experiments *in vitro* we attempted to elucidate the mechanism of activation by endotoxin of the complement system. In many characteristics (activation of the whole complement system, complement fixation at 0 °C) the endotoxin-complement reaction resembles complement fixation by antigen-antibody complexes. The two reactions are very similar in time course. Accordingly, the decreasing effect of endotoxin on the complement level may be due to complement fixation by the reaction between endotoxin and "natural" antibodies present in normal sera.

Data are contradictory as to the role of natural antibodies in the reaction, Kostka and Sterzl [25] and Miler *et al.* [12] showed *in vivo* and *in vitro* that in precolostric piglets free from antibodies, endotoxin causes no decrease in the complement level. Gilbert and Braude [3] observed in the rabbit a decrease in the level of complement and natural haemagglutinating antibodies after intravenous endotoxin injection; frequently, however, there was no correlation between the change in the two titres. The role of antibodies is indicated by the observation of Muschel, Schmoker and Webb [26], which has been confirmed by other authors [11, 12], that in immune serum the same dose of endotoxin causes a higher fall in complement level than in normal serum.

In recent years several data have cast doubt on the role of natural antibodies in the endotoxin-induced decrease in complement titre. Muschel, Schmoker and Webb [26] in disagreement with previous findings observed a higher degree of endotoxin-induced complement decrease in precolostric piglets than in adult animals. The same authors demonstrated that absorption of normal serum by *Salmonella typhi* does not decrease the anticomplementary activity of endotoxin prepared from that organism. Bladen *et al.* reported similar findings after absorption with *Veilonella alcalescens* or its endotoxin. According to Skarnes [8] the lysis of erythrocytes sensitized with endotoxin can be observed even after the absorption of "natural" antibodies from the serum.

Hungarian workers have dealt with the role of antiendotoxins in the Shwartzman phenomenon. According to the hypothesis of KOVÁTS [27], in consequence of a constant symbiosis with endotoxin-producing enteric bacteria, vertebrates are "naturally" hypersensitive to endotoxins. KOVÁTS *et al.* [28] described that skin symptoms constituting the first phase of the Shwartzman reaction highly resemble the changes observed in the Arthus phenomenon. The examinations of KOVÁTS and VÉGH [29] indicate that the Shwartzman phenomenon is immunospecific. In disagreement with this opinion, KESZTYÜS *et al.* [30] state that there is a basic difference in mechanism between the Arthus and the Shwartzman phenomenon. According to KESZTYÜS the Shwartzman reaction cannot be regarded as the result of one process started by a single endotoxic effect; the endotoxin attacks various parts of the body and gives rise to various alterations independent of one another — the summation and integration of these changes occur later [31].

Our results indicate that natural antibodies play no part in the process. No correlation has been found in individual and pooled sera between the titre of natural antibodies and the degree of endotoxin-induced decrease in complement titre.

Two different preparations of *S. marcescens* endotoxin differed considerably in anti-complementary activity despite the fact that the antibody titre of the serum was similar against both endotoxins. It has also been shown that endotoxin decreases the complement level in sera from which the "natural" antibodies have been removed. The fact that the proportion of fixed complement components was the same in absorbed and in unabsorbed sera indicates that the process is independent of the presence or absence of antibodies.

Accordingly, most data in the literature as well as our experiments show that in normal serum the endotoxin-induced decrease of complement is not, or at least not completely, the result of antigen-antibody reaction. In view of these considerations the question arises as to the mechanism of the complement-activating effect of endotoxin. The following explanations may be considered.

1. The endotoxin exerts a direct activating effect on the first component of complement and starts the process of complement fixation. PEARLMAN, SAUERS and TALMAGE [2] denied this mechanism on the basis that the lag phase of the complement-decreasing reaction was prolonged in the rabbit. In dogs no such prolonged lag phase has been demonstrated: the complement level decreased definitely 10 minutes after the endotoxin injection [17].

2. The role of serum properdin may also be considered. PENSKY *et al.* [32] succeeded in preparing highly purified human properdin. By immunochemical experiments they were able to separate properdin from all known immunoglobulins and complement components. The identity of properdin with natural antibodies [24] has not been accepted but, as emphasized by PENSKY *et*

al. [32], there are no sufficient experimental data with purified properdin to decide which of the many effects attributed earlier to the "properdin system" are really due to properdin.

3. Finally, it may be supposed that the plasminogen-plasmin system is involved especially in the *in vivo* complement-decreasing effect of endotoxin. The final elucidation of the problem needs further experimental data.

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INVESTIGATIONS ON THE OLIGOSACCHARIDE DECOMPOSITION BY ENDOMYCOPSIS WICKERHAMII VAN DER WALT

By

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Summary. In the course of oligosaccharide decomposition by the yeast *Endomycopsis wickerhamii* no raffinose splitting enzyme was demonstrated. The sucrose splitting enzyme is not of the invertase type but proved to be similar to or identical with those isolated by us from other yeast. The maltose cleaving enzyme of the investigated species differed from those isolated from other yeasts insofar as it showed acetone sensitivity.

The pathways of carbohydrate utilization of yeasts are important from the taxonomical as well as from the physiological point of view and also in the fermentation industry. As on the basis of theoretical deduction [13, 14, 17] the existence of more than one pathway of sucrose and maltose utilization had been assumed, a series of experiments dealing with the uptake and cleavage of these oligosaccharides was started.

Among taxonomists, the role of invertase in splitting of sucrose had only been accepted [5], before the sucrose splitting capacity of maltase (group-specific alfa-glucosidase) in invertase-less yeasts has been shown [3]. According to Halvorson [2], all of the so called yeast-maltase preparations split sucrose as well. Hestrin and Lindegren [4], however, mentioned an enzyme studied by them in a strain of the *Carbondale pedigree* and found it to be different in at least two respects from invertase and maltase (group-specific alpha-glucosidase) as this sucrose splitting enzyme is located intracellularly and splits neither raffinose nor maltose or melezitose. They quoted an unpublished experiment of C. Raut who investigating the pH function of sucrose cleavage by the enzyme in question found a rather sharp optimum in the pH range 5.8—6.6, different from that of invertase. Unfortunately no further data concerning the characteristics of this enzyme are available. A sucrose splitting enzyme differing from invertase in its cellular localization and inactivity to raffinose was observed by us in *Candida solani* [8] and it was shown not to be identical with "yeast-maltase". On the basis of its lipoid solvent sensitivity a similar enzyme was later isolated by us [15] from *Procandida albicans* — syn. *Candida albicans* [12] — and on the basis of the behaviour of this enzyme against inhibitors, its nature differing from invertase was confirmed [16]. Since then a

similar enzyme has been shown to occur in *Candida requinyii, Procandida stellatoidea* and *Procandida grubyi* [9, 10, 11].

Investigating the occurrence of this enzyme in yeasts, first of all species which utilize sucrose but not raffinose were tested. In the present paper, results concerning the sucrose, maltose and raffinose splitting capacity of *Endomycopsis wickerhamii* are reported. This species assimilates glucose, sucrose and maltose but ferments only glucose. Besides, an interesting comparison is given by the fact that *Procandida grubyi* [11] shows a sugar assimilation pattern which is identical with that of *Endomycopsis wickerhamii* but it ferments besides glucose also maltose.

Materials and methods

For the experiments a strain of *Endomycopsis wickerhamii* received from Dr. VAN DER WALT was cultivated on CSILLAG's molasses agar [1] in Roux bottles. The technique of experiments with intact and acetone treated cells and cell-free extracts as well as the method of paper chromatography have been described earlier [6, 7, 9].

Results

Raffinose splitting. Neither cleavage nor uptake of raffinose was demonstrated by any of the preparations (*e.g.* see the chromatogram with acetone treated cells, Fig. 1).

Maltose splitting. The living and the acetone treated cells neither split nor took up this sugar (as an example, the chromatogram of the living cells is shown in Fig. 2), while in the cell-free extract maltose splitting could be demonstrated (Fig. 3).

Sucrose splitting. The living and the acetone treated cells neither split nor took up sucrose (see in Fig. 4 with living cells), but in the cell-free extract sucrose splitting occurred (Fig. 5).

Discussion

From incubations made with sucrose and raffinose it is seen that the sucrose splitting enzyme of *Endomycopsis wickerhamii* is not of the invertase type, since it does not cleave raffinose and is located intracellularly. Its acetone sensitivity, however, refers to its similarity to, or identity with the enzyme isolated by us from some other yeasts [8, 9, 10, 11, 15, 16].

The maltose splitting enzyme of *Endomycopsis wickerhamii* differs considerably from those isolated by us from other yeasts [7, 8, 10, 11, 15, 16] it being acetone sensitive while the others are not. The yeast in question may thus be supposed to have a different type of maltose splitting enzyme than the species investigated by us earlier.

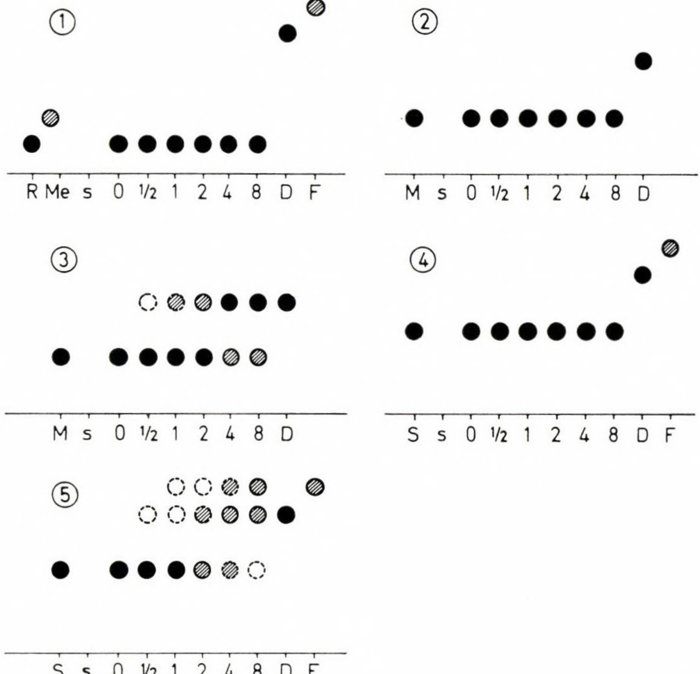

Fig. 1. Raffinose utilization by acetone treated *E. wickerhamii* cells
Acetone treated 750 mg live wet cells and 60 mg raffinose in $M/30$ pH = 7.2 phosphate buffer in 3 ml volume. Chromatogram. Left, raffinose, melibiose and suspension, right, glucose and fructose controls. Figures under the line represent the sampling intervals in hours

Fig. 2. Maltose utilization by living (intact) *E. wickerhamii* cells
750 mg live wet cells and 60 mg maltose in $M/30$ pH = 7.2 phosphate buffer in 3 ml volume. Left, maltose and suspension, right, glucose controls. Others, as indicated in Fig. 1

Fig. 3. Maltose utilization by cell-free extract of *E. wickerhamii* cells
Cell-free extract of 750 mg quartz sand-disintegrated live wet cells and 60 mg maltose in $M/30$ pH = 7.2 phosphate buffer in 3 ml volume. Others, as indicated in Fig. 2

Fig. 4. Sucrose utilization by living (intact) *E. wickerhamii* cells
750 mg live wet cells and 60 mg sucrose in $M/30$ pH = 7.2 phosphate buffer in 3 ml volume. Left, sucrose and suspension, right, glucose and fructose controls. Others, as indicated in Fig. 1

Fig. 5. Sucrose utilization by cell-free extract of *E. wickerhamii* cells
Cell-free extract of 750 mg quartz sand-disintegrated live wet cells and 60 mg sucrose in $M/30$ pH = 7.2 phosphate buffer in 3 ml volume. Others, as indicated in Fig. 4

Comparing the results obtained with living cells of *Endomycopsis wickerhamii* with those obtained with living cells of *Procandida grubyi* [11] it can be established that no maltose consumption was observed even in suspensions of a high density (see Figures), *i.e.* the living cells did not take up maltose. In constrast, in the case of *Procandida grubyi* maltose uptake was demonstrated in suspensions of a lower density too [11]. Accordingly, of the two species

being able to grow on maltose aerobically only that capable of fermenting it anaerobically *(Procandida grubyi)* took up this sugar in a demonstrable amount. Taking into consideration that our incubation method provides practically anaerobic conditions, it is plausible that only the species having an anaerobic maltose metabolism takes up this sugar, and thus results confirm in the case of maltose utilization the existence of two types (anaerobic and aerobic) of transportase systems or enzymes respectively, or more conveniently the inductive nature (= dependence not only on the presence of an inducer but also on aeration) of one type of this system at least by some yeasts.

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OBSERVATIONS ON PRIMARY BACTERIAEMIA*

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Summary. Mouse-virulent *Salmonella enteritidis* and apathogenic *Escherichia coli* O111 : B4 : H2 cultures were administered to mice orally. The results indicated that the development of primary bacteriaemia may not be associated with the ability of the organism actively to penetrate the intestinal mucosa, but depends considerably on the experimental method. The relevant literature has been discussed.

Primary bacteriaemia has long been a problem in mouse salmonellosis [1—13]. The association between primary bacteriaemia and the virulence of the agent, infective dose and mode of infection has not yet been elucidated. In the present study the association between pathogenicity and ability to cause primary bacteriaemia and the influence of the conditions of oral infection have been investigated.

Materials and methods

Organisms. Mouse-virulent *S. enteritidis* and apathogenic *E. coli* O111 : B4 : H2 (resis tant to 5000 μg/ml streptomycin) were used. Both strains were obtained from the collection of our institute.

Animals. The mouse strain of the Institute for Serobacteriological Production and Research "Human" Budapest, was used.

Oral infection. A 20 hour agar slant culture was suspended in saline and adjusted to the density required by comparing to opacity standards. The suspension was injected into the stomach of the animal at 0.5 or 0.1 ml amounts with a catheter fitted to a syringe.

Autopsy and bacteriological examination. The mice were sacrificed by damaging their medulla. The skin, then the pectoral and abdominal wall were disinfected with iodine. After opening the pleural cavity heart blood samples were taken with a sterile capillary. Then the abdominal cavity was opened and the spleen and part of the liver were removed. Each step of the procedure was performed with separate sterile instruments. The heart blood and organs were placed separately in tubes containing broth. After 24 hours incubation the organs were crushed with sterile glass rods and the tubes were incubated for another day. After platting on Endo agar the organisms were identified by the usual methods.

Results

In the first step of the examinations two groups of mice each comprising 55 animals were infected with 0.5 ml aliquots of suspensions representing 5×10^8 cells of virulent *S. enteritidis* and apathogenic *E. coli*. Results are shown in columns 1 and 2 of Table I.

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In 4 mice *S. enteritidis* was cultured from all three kinds of specimen. In 11 mice only one or two kinds of specimen were positive for this organism (10 liver, 9 spleen and 9 heart blood cultures). From 40 animals no salmonellae were recovered. Among mice infected with *E. coli* all specimens were positive for 3 animals. The distribution of positive cultures was as follows: 4 heart blood, 11 liver and 9 spleen specimens. Negative results were obtained for 42 mice.

Table I

Recovery of S. enteritidis and E. coli O111 : B4 : H2 from organs of mice infected orally

Organs	Number of mice giving positive culture after infection with					
	1 *S. enteritidis* 5×10^8 cells in 0.5 ml	2 *E. coli* 5×10^8 cells in 0.5 ml	3 *S. enteritidis* 5×10^8 cells in 0.5 ml	4 *E. coli* 5×10^8 cells in 0.5 ml	5 *E. coli* 5×10^5 cells in 0.5 ml	6 *E. coli* 5×10^5 cells in 0.1 ml
Heart + liver + spleen	4	3	2	—	—	—
Heart + liver	1	—	—	—	—	—
Heart + spleen	3	1	1	1	1	—
Liver + spleen	1	3	3	5	1	—
Heart	1	—	—	1	—	—
Liver	4	4	3	3	2	—
Spleen	1	2	1	—	—	1
Time in minutes between infection and autopsy	10	10	20	20	10	10
Total no. of positive mice	15	13	10	10	4	1
No. of negative mice	40	42	30	30	46	49

Although the two strains differed considerably in pathogenicity, there was no significant difference in their incidence in the various specimens.

The next experiment was carried out on two groups of 40 animals each. The only difference from the first experiment, in which the organs were cultured 10 minutes after infection, was that the animals were sacrificed 20 minutes after administration of the test organisms. Results are shown in columns 3 and 4 of Table I. In each of the two groups the infective agents were recovered from 10 animals. In mice infected with *S. enteritidis* 3 heart blood, 8 liver and 7 spleen cultures, in those receiving *E. coli* 2 heart blood, 8 liver and 6 spleen cultures were positive.

Similarly to the result of the first experiment, the difference was not significant statistically. Accordingly, on the basis of earlier and the present findings, it has been concluded that primary bacteriaemia occurring after artificial oral infection is not associated with the pathogenicity or virulence of the organism.

As the results were identical for *S. enteritidis* and *E. coli*, subsequent experiments were carried out only with the latter organism. In order to exam-

ine whether the infective dose influences the incidence of primary bacteriaemia, 50 mice were infected with 0.5 ml aliquots of suspension representing 5×10^5 cells. Results are demonstrated in column 5. The bacteria were recovered only from 4 mice (1 heart blood, 3 liver and 2 spleen specimens), thus the number of positive cultures was smaller than in the first experiments.

In the fourth experiment the influence of the volume of the bacterial suspension was examined. The 0.5 ml dose used in the above experiments seemed to be too large for one mouse — in man it would correspond to a 1.5 litre oral intake of fluid. The results obtained with 0.1 ml volumes of *E. coli* suspension are shown in column 6. Out of 50 mice only one yielded a positive spleen culture; the organs of the remaining 49 mice were sterile. This finding indicates that the appearance of primary bacteriaemia is influenced by both the absolute number of cells and the volume of the suspension.

Discussion

In examining the pathomechanism of mouse salmonellosis only part of the authors recognize the existence of primary bacteriaemia.

According to Müller [1] primary bacteriaemia occurs under symptomless conditions as the first phase of the infection. In his opinion only strains exhibiting a maximum degree of virulence are able to invade the blood stream from the intestinal tract. He applied large doses of the infective agent. Elkeles [2] was of the same opinion. Seiffert [3] demonstrated salmonellae in small numbers some hours after infection in the blood, lymph nodes and visceral organs. Kligler and Olitzki [4] showed the test bacteria within 24 hours in the organs of some of their animals infected with large doses (10^9 cells in 0.05 ml volume). Despite this finding they claimed that the existence of primary bacteriaemia could be excluded and the bacteria invaded the blood stream *via* the lymphatic system. Prior to their observations a similar conclusion had been drawn by Danish authors [5—7] who were unable to demonstrate primary bacteriaemia. Lange and Yoshioka [10] were of the same opinion. Gerichter [11], in order to throw light on the contradictory findings, infected mice with *S. typhi*, *S. paratyphi-A* and *S. paratyphi-B*. He observed primary bacteriaemia 20 to 30 seconds after the ingestion of these non-mouse-virulent organisms. He supposed that it was through the portal veins that the bacteria entered the blood stream as they were frequently present in the liver. Gerichter observed a direct relationship between the number of bacteria and the incidence of primary bacteriaemia, but found that the volume of the suspension exerted no influence. Rauss *et al.* [12] observed early bacteriaemia after oral infection with virulent *S. enteritidis* and they showed a similar phenomenon in the case of avirulent salmonellae, too. Morello *et al.* [13] fed mice with 0.5 ml of a suspension containing 10^9 cells and recovered both avirulent

and virulent salmonellae from the blood and organs in some of the mice 6 hours after infection. Ingestion of 10^6 cells produced no such manifestation. McGuire and Floyd [14, 15] showed that in experimental shigellosis the test organism could be recovered from the blood of one third of mice infected with 10^8 cells, although it is generally accepted that shigellae have no invasive power under the conditions applied. According to Raettig [16, 17] phages and viruses are able to enter the blood stream from the intestinal tract. The phenomenon is not associated with the pathogenicity of viruses and can be demonstrated some minutes after the ingestion of the agent. After oral administration of labelled *S. typhi-murium* to mice, radioactivity in the blood increased although killed cells were given. Furuta *et al.* [18] showed *E. coli* in the organs of a small part of mice infected orally. Hildebrand *et al.* [19] observed that the number of bacterial cells or virus particles in the lymph of rats depends on the number ingested. If the mucosa is intact, only few test organisms enter the lymphatic system. Hildebrand *et al.* failed to show the agents in portal blood. Wolochow *et al.* [20] emphasized that the number of microorganisms entering the lymphatic system as a result of a "non-infective process" was usually low and was influenced by several factors.

It is evident from the presented data that the existence of primary bacteriaemia is a debated problem. From our experiments the following conclusions can be drawn. Both pathogenic and apathogenic bacteria may enter the blood stream and visceral organs after oral infection. The phenomenon may not be associated with pathogenicity or virulence. A direct relationship seems to exist between the number of infective cells and the volume of the bacterial suspension. It follows that the so-called primary bacteriaemia cannot be part of the mechanism of the infection in the usual sense. According to some authors, primary bacteriaemia is a result of a direct active invasion from the intestinal tract by only virulent [1, 2, 12] or also by avirulent [11] organisms. All investigators who demonstrated this phenomenon had infected their animals with high doses (mainly in the range of 10^9 cells). All authors except Müller [1] and Elkeles [2] used catheters for infecting the mice using thus a method not resembling natural infection. Last but not least, all the authors except the above cited and Kligler *et al.* [4] introduced the bacteria into the stomach with 0.5 ml of fluid. This volume seems to be too large for a mouse and it may cause an abnormal intra-intestinal pressure. This change might play a role in the induction of primary bacteriaemia as well as in the other differences existing between artificial and natural infection. This has also been pointed out by Waldmann and Rostowa [21]. At present the role of these factors is not yet exactly known. The bacteria may penetrate the intestinal wall independently of the effect of pressure and volume of ingested vehicle fluid but depending on the number of cells ingested and the process may be independent of any particular active penetrating capacity. The observa-

tions of Raettig [17] support this consideration. Penetration of the organisms may theoretically be promoted by persorption [22] and perhaps by phagocytosis. In that phase of the infection the virulence factor (ability to multiply in the tissues) may play a role by determining the number of bacterial cells present in the intestines and intestinal wall. The permeability of the intestine and the penetrating capacity of bacteria await further investigations.

On the basis of our findings [12, 23] we are of the opinion that the concept of Danish authors [5—9] is correct in that the salmonellae first enter the intestinal wall where they multiply and then they invade the blood stream and viscera *via* the lymph vessels. In this manner bacteriaemia develops which, in infections with virulent strains, becomes more and more grave and the animal dies. After infection with an avirulent strain an equilibrium without bacteriaemia develops.

These hypotheses are supported by the observations of Sprinz *et al.* [24], Gaines *et al.* [25] and Tully *et al.* [26—28] in chimpanzees and of Schubart *et al.* [29] in human volunteers infected artificially with *S. typhi*. These authors demonstrated that bacteriaemia developed not immediately after the infection but in later stages of the disease.

Our results may throw some light on bacteriaemia in food-borne infections observed in some, but not all patients in the acute stage. Among many other factors the number of cells ingested may play a role in this phenomenon.

If it is true that there is no active penetrating power, as it was imaginable on the basis of our results, our data would explain why large amounts of "facultatively pathogenic" bacteria may cause symptoms of food poisoning.

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NEW OBSERVATIONS ON ORAL VACCINATION AGAINST DYSENTERY

By

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Summary. (i) A high mouse-protective antibody level induced in adults by parenteral vaccination with adsorbed dysentery vaccine was prolonged during an observation period of 6 months by oral administration of the 5-fold parenteral dose (about 0.5 mg dry Boivin antigen for each component) at intervals of one or two weeks. When given at intervals of three or four weeks the same dose of vaccine failed to maintain sufficient immunity.

(ii) The immune response to oral revaccination of children who had received either one or two doses of adsorbed vaccine as basic immunization was similar to that of adults.

(iii) The results have indicated that, considering the safety limit, routine revaccinations should be performed at 10-day intervals.

(iv) As shown by mouse-protective antibody level determinations, an effective basic immunity was induced in adults with two oral doses representing a 30-fold parenteral dose (about 3 mg dry Boivin antigen for each component). Immunity was maintained by oral revaccinations carried out at intervals of not more than 14 days. Without revaccination the antibody titres decreased rapidly.

(v) Children developed mouse-protective antibodies less readily after oral basic immunization with doses corresponding to, or higher than, those given to adults. After revaccination the titres rose satisfactorily. This finding was attributed to the beneficial effect of prolonged antigen stimuli.

(vi) Uniformly high antibody titres were shown at least for two months after the oral revaccination course regardless whether basic immunization had been performed parenterally or orally. This observation again indicated the importance of prolonged small antigen stimuli.

(vii) The advantage of Boivin antigen over corpuscular vaccines and the importance of epidemiological observations for estimating the value of laboratory results in vaccination against infectious diseases have been pointed out.

It has been shown previously [1] that high titre mouse-protective antibodies produced in human beings after subcutaneous injection of adsorbed typhoid-tetanus-dysentery vaccine [2] can be maintained by oral revaccination* with Shigella antigens in doses found effective in animal experiments [3].

In view of this finding it seemed desirable to study the effect of small oral booster doses administered at different intervals. The effectiveness of oral revaccination indicated that a basic immunity could probably be induced by giving the antigen by mouth. The immune response of children to oral vaccination was also to be elucidated. The present paper gives an account of investigations into these problems.

* The term "revaccination" refers in this paper to a prolonged oral administration of the antigens in order to maintain a basic immunity induced by parenteral or oral vaccination.

Materials and methods

Vaccinated persons. A total of 200 adult volunteers and 605 children aged 5 to 15 years were examined.

Parenteral immunization. Adsorbed typhoid-tetanus-dysentery vaccine elaborated previously [2] was used. In addition to tetanus toxoid it contained Boivin extracts of *S. typhi*, *Sh. flexneri* 2a, *Sh. flexneri* 3 and *Sh. sonnei* adsorbed in the optimal adjuvation zone [4] to $Al(OH)_3$ gel as modified by RÉTHY [5]. Two subcutaneous injections were given at intervals of 4 weeks.

Oral vaccination. Freeze-dried antigens prepared from Boivin extracts precipitated with 4 volumes of ethanol were tabletted [1]. Each tablet contained a 5-fold amount of the parenteral dose, that is, 50 haemagglutination-inhibiting units (about 0.5 mg dry antigen) for each component (*Sh. flexneri* 2a, 3, *Sh. sonnei*). Antigen-free placebo tablets were used as controls. The tablets were given in the morning approximately 1/2 hour before breakfast. The schedule for oral vaccination and revaccination will be detailed in subsequent parts of this paper.

Blood samples were taken before parenteral or oral basic vaccination, after basic vaccination (before revaccination) and after revaccination as detailed below. Individual sera from each group were mixed at equal volumes and stored in the deep freezer.

Mouse protection test. Graded amounts of the mixed serum samples were given subcutaneously to groups of mice each consisting of 4 to 5 animals [1, 3]. The mice were challenged 18 hours after immunization with *Shigella* cultures suspended in 5% mucin. The results were read after 3 days observation.

Statistical evaluation. LD_{50} and ED_{50} values were estimated as described by KÄRBER [6]. In order to simplify comparison, the relative potency (RP) was calculated by considering the pre-immunization titre as 1. Significance of the differences was analysed by the χ^2 method at the 5% level.

Results

1. Adults receiving subcutaneous basic immunization and oral revaccination. In order to supplement our previous data [1] we examined the needed frequency of oral revaccination, *i.e.* the longest interval optimal for maintaining immunity induced by parenteral immunization. The duration of immunity prolonged by oral booster doses was also studied.

The volunteers were divided into groups of 30 individuals. All persons were given two subcutaneous injections of adsorbed vaccine, then four weeks after the second injection oral revaccination was begun as follows. One vaccine tablet was administered at intervals of one week to group I, two weeks to group II, three weeks to group III and four weeks to group IV; members of group V received placebo tablets. Revaccination lasted for 5 months. Mouse-protective antibody titres were examined before basic immunization, four weeks after the second injection (before revaccination), two weeks after finishing revaccination (6 months after basic immunization) and two months after finishing revaccination (eight months after basic immunization).

The result of passive protection tests for mice challenged with *Sh. flexneri* 2a is presented in Table I.

It is evident that the high protective antibody level induced by the adsorbed vaccine was maintained when revaccination was administered at intervals of one or two weeks. Booster doses given at intervals of 3 or 4 weeks were insufficient: the decrease in titre was similar to that observed in the placebo groups.

Table I

Mouse-protective potency against Sh. flexneri 2a of sera from adults given adsorbed vaccine subcutaneously as basic immunization and oral revaccination over a period of 5 months
(Challenging dose: 200 LD_{50})

Time of taking serum samples	Groups; revaccination at intervals				
	I 1 week	II 2 weeks	III 3 weeks	IV 4 weeks	V Not revaccinated
	ED_{50} and RP*				
Before immunization	0.016 (1)**				
1 month after basic immunization	0.0005 (32)	0.0005 (32)	0.0005 (32)	0.0005 (32)	0.0005 (32)
2 weeks after revaccination (6 months after basic immunization)	0.0016 (10)	0.0009 (17)	0.009 (1.7)	0.009 (1.7)	0.016 (1)
2 months after revaccination (8 months after basic immunization)	0.0005 (32)	0.0009 (17)	0.028 ($\sim$1)	0.028 ($\sim$1)	0.016 (1)

* Bracketed figures indicate relative potency.
** Average for revaccinated and not revaccinated groups.

As mouse-protection tests with *Sh. flexneri* 3 and *Sh. sonnei* gave similar results, it was justified to average the relative potencies (Table II).

From the average relative potencies indicating the effectiveness of immunization it may be concluded that revaccination at weekly or two-weekly intervals maintained the original mouse-protective antibody level during the 5-month observation period.

Table II

Average relative mouse-protective potency against Sh. flexneri 2a, 3 and Sh. sonnei of sera from adults given adsorbed antigen subcutaneously as basic immunization and oral revaccination over a period of 5 months

Time of taking serum samples	Groups; revaccination at intervals				
	I 1 week	II 2 weeks	III 3 weeks	IV 4 weeks	V Not revaccinated
	Average RP values				
Before immunization	1*				
1 month after basic immunization	24	22	24	26	22
2 weeks after revaccination (6 months after basic immunization)	16	14	1.7	2.2	1.4
2 months after revaccination (8 months after basic immunization)	17	11	1.4	1.2	1

* Average for revaccinated and not revaccinated groups.

From Table II it is also clear that the same doses administered at intervals of three or four weeks failed to maintain the level; in fact, the relative protective potency found for these groups showed no difference from that for the placebo group. In accordance with observations to be presented, it was clear that oral revaccination prolonged the immunity for a considerable period after revaccination had been discontinued: fairly high titre mouse-protective antibodies were demonstrated eight months after the second parenteral immunization. These findings indicate that observing the proper interval of stimuli is important not only for maintaining but also for prolonging the immunity.

2. Adults receiving oral basic immunization and oral revaccination. Two groups were formed: as a basic immunization each member of group A received 5 tablets in one dose (25-fold parenteral dose, about 2.5 mg dry antigen for each component). Members of group B received first 3 tablets then after a one week interval 3 tablets again (2×15-fold parenteral dose, about 2×1.5 mg antigen).

One week after the basic immunization, members of both groups were given one tablet weekly as a booster dose. The control groups received placebo tablets.

Blood samples were taken one week after basic immunization in group A and one week after each dose in group B. One week after revaccination (5 weeks after basic immunization) and 5 weeks after revaccination (10 weeks after basic immunization) the volunteers were sampled again.

Mouse-protective titres of the serum samples against *Sh. flexneri* 2a are presented in Table III.

It is seen that oral basic immunization resulted in an immunity identical in order of magnitude with that obtained in the subcutaneously vaccinated control group. Basic immunization with 2×3 tablets seemed to be more effective although the difference from group A was not significant (Table IV). Table III indicates also that the mouse-protective antibody level was maintained by weekly oral revaccinations but fell rapidly in not revaccinated persons.

As mouse protection tests for *Sh. flexneri* 3 and *Sh. sonnei* gave identical results, the relative potencies of the sera for the three test strains were averaged (Table IV).

Table IV demonstrates that the average mouse-protecting potency increased after a single dose of 5 tablets 10.8 times while after two doses each comprising 3 tablets, 19.3 times. Although in the resulting antibody titre there was no significant difference between single-dose and two-dose vaccination, it would appear that the latter was more effective. Accordingly, smaller repeated stimuli are preferable to a single large one. A more convincing difference of the same kind was revealed in children (see below).

The effectiveness of revaccination was clearly demonstrated by the fact that doses of one tablet weekly maintained the antibody titres at a level reached

Table III

Mouse-protective potency against Sh. flexneri 2a of sera from adults vaccinated and revaccinated orally over a period of 4 weeks
(Challenging dose: 75 LD_{50})

Time of taking serum samples	Group A		Group B	
	Basic immunization			
	1×5 tablets		2×3 tablets	
	Revaccinated	Not revaccinated	Revaccinated	Not revaccinated
	ED_{50} and RP*			
Before immunization**	0.05 (1)		0.05 (1)	
1 week after the first dose of 3 tablets**	.		0.003 (17)	
1 week after complete basic immunization (before revaccination)**	0.003 (17)		0.0016 (31)	
1 week after 4 doses of revaccination (5 weeks after basic immunization)	0.003 (17)	0.016 (3)	0.003 (17)	0.028 (1.7)
5 weeks after revaccination (10 weeks after basic immunization)	0.003 (17)	0.028 (1.7)	0.0016 (31)	0.028 (1.7)
Control: 4 weeks after adsorbed vaccine injection	0.005 (10)			

* Bracketed figures indicate relative potency.
** Average for revaccinated and not revaccinated groups.

after basic immunization, whereas in the placebo group the titre decreased significantly (P = 0.05) below the immunity level. It was striking that five weeks after the discontinuation of revaccination ($2^1/_2$ months after basic immunization) the protective titres remained at maximum levels and showed a statistical homogeneity as compared to the result of the control subcutaneous immunization with adsorbed vaccine (P = 0.99) similarly to the immunological state observed after parenteral basic and oral booster vaccination.

3. Children receiving subcutaneous or oral basic immunization and oral revaccination. In a community, 525 children aged 5 to 15 years were divided at random into 3 groups of 175. Group I was immunized with two doses of 0.5 ml adsorbed typhoid-tetanus-dysentery vaccine at intervals of four weeks. Group II received a single injection of 0.5 ml vaccine. Group III was given two oral doses each comprising 3 tablets at 5-day intervals. Oral revaccination was commenced four weeks after basic immunization in Groups I and II and one week after basic immunization in Group III. All three groups were subdivided into five subgroups of 35. Each member of every subgroup received one tablet as a revaccination dose at intervals of 1, 2, 3 and 4 weeks, respectively; the fifth subgroup served as a not revaccinated control. Blood samples were taken four weeks after parenteral basic immunization and one week after the second

Table IV

Average relative mouse-protective potency against Sh. flexneri 2a, 3 and Sh. sonnei of sera from adults vaccinated and revaccinated orally over a period of 4 weeks

Time of taking serum samples	Basic immunization			
	1×5 tablets		2×3 tablets	
	Revaccinated	Not revaccinated	Revaccinated	Not revaccinated
	Average RP values			
Before immunization	1		1	
1 week after the first dose of 3 tablets			7.7	
1 week after complete basic immunization (before revaccination)	10.8*		19.3*	
1 week after 4 doses of revaccination (5 weeks after basic immunization)	10.8**	2.6**	14.7	2.6
5 weeks after revaccination (10 weeks after basic immunization)	12.3***	1.2	17.0	1.9
Control: 4 weeks after absorbed vaccine injection	15.1***			

χ^2 analysis: * = 0.503 (P = 0.5); ** = 3.839 (P = 0.05); *** = 0.056 (P = 0.99).

dose of oral basic immunization. The effect of revaccination was checked one and four weeks after the discontinuation of revaccination (17 or 13 weeks and 21 or 17 weeks after basic immunization, respectively). The serum samples were pooled according to subgroups and were tested for mouse-protective potency.

The average relative potencies against *Sh. flexneri* 2a, 3 and *Sh. sonnei* are summarized in Table V.

The effect of two subcutaneous injections was essentially identical with that observed in adults (see Table II). One single dose gave also rise to a sufficient immune response statistically homogeneous with the titre for the two-dose group. The result of revaccinations was also similar to findings in adults. Revaccinations administered at intervals of one week or even of two weeks maintained the protective antibody titres at high levels; when, however, the booster doses were given at intervals of more than two weeks, the titres fell to the range observed in the not revaccinated control group. The persistence of effective immunity for four weeks after revaccination (21 weeks after basic immunization) in children revaccinated at weekly or two-weekly intervals was also evident (compare with Table II).

It is interesting that the immune response in the oral group of children was considerably weaker than in adults treated in a similar manner (see data in Table IV). Adults given 2×3 tablets showed a 19.3-fold increase in relative

Table V

Average relative mouse-protective potency against Sh. flexneri 2a, 3 and Sh. sonnei of sera from children given one or two doses of adsorbed vaccine subcutaneously or oral antigen as basic immunization and revaccinated orally over a period of 12 weeks

Group	Basic immunization	Intervals of oral revaccinations (weeks)	Average RP values			
			Before basic immunization	After basic immunization (before revaccination)	1 week after revaccination	4 weeks after revaccination
I	Adsorbed vaccine 2× subc.	1			17.8[c]	.
		2			12.4	.
		3	1	34[a]	3.9	.
		4			2.6	.
		Not revaccinated			3.1	.
II	Adsorbed vaccine 1× subc.	1			13.1[c]	14.7[d]
		2			17.0	10.0
		3	1	24[a]	6.2	4.9
		4			3.4	1.7
		Not revaccinated			1.7	1
III	Oral vaccine 2×3 tablets	1			10.8[e]	6.1[c]
		2			7.7	3.2
		3	1	4.9[b]	1.9	1.3
		4			1.2	1.2
		Not revaccinated			1.2	1

4 weeks (a), 1 week (b), 17 weeks (c), 21 weeks (d), 13 weeks (e) after basic immunization.

potency while among children the increase was four times less (4.9). After receiving weekly doses for 12 weeks, however, the relative potency increased to 10.8, approaching the titres observed in revaccinated adults. If the effect of vaccination is analysed for individual components of the vaccine it is seen that the lowest rise of protective antibodies was found for *Sh. sonnei* (1.7-fold). Revaccination resulted in a 5.5-fold rise of antibodies against this organism. The most satisfactory immune response was observed against *Sh. flexneri* 3 (10-fold rise before and 17-fold rise after revaccination). *Sh. flexneri* 2a occupied a medium position (3.1-fold rise before and 10-fold rise after revaccination). Revaccinations performed at intervals of two weeks caused a small but definite rise in protective titres; if, however, the booster doses were given at intervals of three or four weeks, similarly as in the control group, the titres fell below the levels observed after basic immunization. Four weeks after

finishing the revaccination course (17 weeks after basic immunization) the degree of immunity showed a similar distribution, *i.e.* the highest protective potency was revealed in the group revaccinated weekly (6-fold rise), while the titres for persons revaccinated at intervals of two weeks fell below the level of basic immunity; in groups receiving the booster doses at longer intervals the titres were in the range shown before immunization.

In subsequent experiments the immune response to 2 doses, each comprising 3 or 5 tablets, was compared in two small groups. In both groups the basic titres rose to levels demonstrated in detail in the above experiments. The next two groups given 3×5 or 4×5 tablets showed no higher rise in basic titre. In view of the results of earlier examinations these groups were revaccinated with 6 doses given at 10-day intervals. Protective antibody titres rose similarly as in the previous experiments. Basic immunization with 2×5 tablets gave better results than with 2×3 tablets, but no further increase in titre was observed after giving 3×5 or 4×5 tablets.

Though we are unable to explain the difference in immune response between adults and children, the findings indicated again that prolonged small antigen stimuli were more effective than larger doses given at shorter intervals. Elucidation of the seemingly different immune mechanism in children and in adults awaits further investigations. Nevertheless, on the basis of mouse-protective antibody titre determinations it may be stated that in children an effective immunity can be produced by the prolonged administration of oral vaccine. A prolonged dosage is necessary not only for maintaining the antibody level but also for increasing the immune response.

Discussion

One purpose of the investigations presented here was to supplement our previous data [16] for the optimal intervals of oral revaccination — after establishing subcutaneously a basic immunity — with antigen doses found effective in earlier experiments (50 haemagglutination-inhibiting units for each component = about 0.5 mg dry antigen). The other purpose was to study whether or not oral vaccination produced a sufficient basic immunity. Both kinds of investigation were performed on adults and children.

In the adult groups receiving adsorbed vaccine as a basic immunization, revaccination was carried out for five months at different intervals. It was found that the maximum protective titre reached after basic immunization could be maintained with oral revaccinations performed at intervals of one or two weeks. The level of protective antibodies in the control (not revaccinated) group fell during this period to titres demonstrated before basic immunization. It was also observed that in persons revaccinated at intervals of 3 or 4 weeks the average protective titres also fell to the level of the control. The

failure of the latter method of revaccination may be explained partly by a reduction in the number of antigen stimuli, partly by the length of the period elapsing between the stimuli. The great importance of the proper intervals between antigen stimuli will be pointed out later in this paper. Thus taking into consideration the safety limits and the, though not significant, tendency to a decrease of immunity in persons revaccinated at 14-day intervals, in routine immunization the oral doses should be given at 10-day intervals.

The persistence of immunity in the successfully revaccinated groups was remarkable. There was no decrease in mouse-protective antibody titres eight months after basic immunization; this was probably due to the effectiveness of prolonged immunization with frequent antigen stimuli.

Children were examined for the time course of immune response to one and two subcutaneous injections of adsorbed vaccine and for the effect of oral revaccination. Both adsorbed and oral vaccines were given in doses identical with those administered to adults. It has been shown that, regardless whether one or two injections had been given, the protective antibody titre was similar to that obtained in adults, *i.e.* a single subcutaneous injection yielded a sufficient basic immunity. There was no difference between children and adults in the result of revaccination. Booster doses given at intervals of one or two weeks maintained the antibodies at levels reached after basic immunization. In contrast, revaccinations at intervals of three or four weeks resulted in a fall of the titres to the level demonstrated in the control group or before basic vaccination. As in the group revaccinated at 14-day intervals the protective antibody titres tended to decrease, a period of ten days has been considered the optimal interval of revaccination. The long duration of immunity in groups immunized successfully was observed in both children and adults. The protective antibody titres remained at high levels for at least 30 days after revaccination.

Oral basic immunization yielded positive results in the adult group. In mouse-protective antibody titres there was no difference from groups vaccinated parenterally with adsorbed antigen. Basic immunization was performed with a single dose of 5 tablets or with two doses of 3 tablets given at a 7-day interval. Although there was no significant difference in effectiveness between the 1×5 and 2×3 tablet doses, the latter tended to produce a more satisfactory immunity. The dose of 2×3 tablets represented a 30-fold amount of the parenteral dose (about 3 mg dry antigen per components). As shown in mice [3] the above doses of oral and parenteral vaccine are immunologically equivalent. Five tablets represented a 25-fold amount of the parenteral dose (about 2.5 mg dry antigen per components). Accordingly, it is improbable that the difference in antibody response should have been due to this small difference in doses; the beneficial effect of repeated stimuli is much more likely.

The rapid fall in the titres of individuals not revaccinated was observed in both groups; five weeks after basic immunization the protective potency was not higher than the normal titre. In contrast, weekly revaccination with the mentioned dose resulted in the maintenance of antibody levels reached after basic immunization. It was remarkable that mouse-protective antibodies were present in a maximum titre 5 weeks after finishing the revaccination course not only in persons given parenteral but also in persons given oral basic immunization. This must have been due to the effectiveness of prolonged antigen stimuli.

Oral basic immunization of children yielded different results. The immune response of children to 2×3 tablets, a dose sufficient for adults, was remarkably weak. However, under the effect of weekly revaccinations the titres rose and finally reached the levels shown in adults after revaccination. The immune response was less satisfactory when revaccination had been performed at intervals of two weeks, and totally ineffective when the booster doses had been given at intervals of three or four weeks. In the latter groups the average titres corresponded in order of magnitude to that demonstrated in the control groups or before basic immunization. Accordingly, a similar rule is valid for the intervals of oral revaccination in both adults and children. A prolongation of protective antibody titres could also be demonstrated: one month after revaccination the titres were considerably high. A 2-week interval between revaccinations had an unfavourable influence on the duration of the immune response: the titres fell below the levels reached after basic immunization.

The experiments were repeated in smaller groups, giving 2×3 or 2×5 tablets. Both doses raised the original titres in an order corresponding to that observed in the first experiments. Administration of 3×5 or 4×5 tablets yielded no higher increase. Revaccination — in these experiments at 10-day intervals — raised the titres with the same regularity as that observed in the first examination: the protective potency was somewhat higher after 2×5 tablets; larger doses (3×5 or 4×5 tablets) ensured no further improvement.

It may be concluded that, as demonstrated in mouse-protection tests, children can be immunized orally as successfully as adults. There is no explanation at present as to the difference between adults and children in the development of immune response. The increase in the level of protective antibodies during revaccination may be attributed to the effect of repeated antigen stimuli. This consideration is supported by the fact that after prolonged revaccination the mouse-protective antibody level persists in both adults and children for at least two months independently of the mode of basic immunization. As findings in children indicate, revaccination is essential not only for maintaining but also for increasing immunity.

The present studies confirmed previous findings [9] as to the excellent antigenicity of colloidal Boivin extract. Doses of 2×3 tablets used for oral

basic immunization represented the Boivin extract of about 9×10^{10} *Shigella* cells for each component. Revaccination was carried out with doses representing about 1.5×10^{10} cells for each antigen. As a comparison it may be mentioned that MEL *et al.* [7] immunized humans with 2.5×10^{11} living streptomycin dependent cells for each serotype and FORMAL *et al.* [8] immunized monkeys with the same dose of living avirulent or with 5×10^{10} living semi-virulent shigellae. The advantage of Boivin antigen is evidently due to dimensional differences ensuring a more favourable absorption of antigen extracts. The present investigations have revealed an important fact, *viz.*, that in immunogenicity the Boivin antigen is equivalent with FORMAL's living semi-virulent culture and is superior to avirulent strains [9]. Consequently, Boivin antigens induce an optimal immune response without the danger arising from the use of live vaccines. RAETTIG, on the basis of his own and other authors' data, has also emphasized that oral vaccines prepared from killed bacteria should given preference over the dangerous live dysentery vaccines [10].

Finally, let us emphasize the importance of epidemiological observations for estimating the value of laboratory results in testing the effectiveness of vaccination against infectious diseases.

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SELECTIVE IN VITRO ANTIVIRAL EFFECT ON MYXOVIRUS STRAINS OF GUANIDINE DERIVATIVES

By

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Summary. The antiviral activity of guanidine and its 16 derivatives on PR8 strain of the influenza A0 virus and on the Sendai strain of parainfluenza 1 virus was examined in surviving chorioallantoic membrane fragments. Some relation was demonstrated between the chemical structure and the antiviral effect of the derivatives.

The antiviral action of guanidine has been known since 1961 [1]. Guanidine inhibits the multiplication of numerous picornaviruses *in vitro,* being most active against polioviruses [2]. It has been shown that a Herpes simplex (HS) virus strain [3] and measles virus [4] are also sensitive to guanidine and the HS and vaccinia viruses are inhibited by certain guanidine derivatives [5, 6].

PÁCSA *et al.* [7] reported on the antiviral action of N-substituted guanidine derivatives on polio, echo, coxsackie and HS viruses. In the present investigations the antiviral action of guanidine and of some of its derivatives was studied *in vitro.* The PR8 strain of influenza A0 virus and the Sendai strain of parainfluenza 1 virus were used as test viruses.

Materials and methods

Cell cultures, culture media, maintenance and storage of viruses, and the methods of determination of toxic and antiviral effects of viruses have been described previously [8, 9].

Results and discussion

From the data in Tables I and II the following conclusions may be drawn.

(1) There is no appreciable difference between the two virus strains in their sensitivity to the compounds under study.

(2) On the basis of the quotient + + toxic dose/lowest effective dose compounds H-904, H-900, H-901 and V-53 proved to be most selective; V-130 and V-144 proved to be less effective than guanidine, and V-139 and V-140 were ineffective.

Table I

The compounds tested

Designation	Chemical structure	Molecular weight
Guanidine HCl	$NH_2-C(=NH)NH_2 \cdot HCl$	95.575
H-900	$[\text{2-pyridyl}-CH_2-NH-C(=NH)NH_2]_2 \cdot H_2SO_4$	398.45
H-902	$[\text{4-pyridyl}-CH_2-NH-C(=NH)NH_2]_2 \cdot H_2SO_4$	398.45
H-903	$[\text{3-pyridyl}-CH_2-NH-C(=NH)NH_2]_2 \cdot H_2SO_4$	398.45
H-901	$[\text{6-methyl-2-pyridyl}(CH_3)-CH_2-NH-C(=NH)NH_2]_2 \cdot H_2SO_4$	426.52
H-904	$[\text{2-pyridyl}-CH_2-N(CH_3)-C(=NH)NH_2]_2 \cdot H_2SO_4$	426.52
H-540	$[\text{phenyl}-CH_2-NH-C(=NH)NH_2]_2 \cdot H_2SO_4$	396.45
H-539	$\text{furyl}(O)-CH_2-NH-C(=NH)NH_2 \cdot 1/2H_2SO_4$	188.20
PYG (V-53)	$\text{2-pyridyl}-CH_2-CH_2-NH-C(=NH)NH_2$	165.224
H-915	$[\text{4-pyridyl}-CH_2-CH_2-NH-NH-C(=NH)NH_2]_2 \cdot H_2SO_4$	456.535
V-130	$\text{2-chlorophenyl}(Cl)-NH-C(=NH)NH_2 \cdot HCl$	206.072

Table I (continued)

Designation	Chemical structure	Molecular weight
V-137	HO (meta)–(benzene ring)–NH–C(=NH)–NH$_2$ · HCl	187.64
V-138	HO–(benzene ring)–NH–C(=NH)–NH$_2$ · HCl	187.64
V-139	O_2N (meta)–(benzene ring)–NH–C(=NH)–NH$_2$ · HCl	216.64
V-140	(benzene ring)–NH–NH–C(=NH)–NH$_2$ · HCl	186.67
V-144	(benzene ring, ortho NH$_2$ · HCl)–NH–C(=NH)–NH$_2$ · 2HCl · H_2O	241.12
V-145	H_2N–(benzene ring)–NH–C(=NH)–NH$_2$ · HCl	186.647

(3) Substitution by a pyridine ring (H-900) of the benzol ring in H-540 or of the furane ring in H-539 increases the activity of the compound.

(4) The position of the side-chain of the pyridine ring is of importance. A side-chain at site 2 (H-900) is more favourable than the same side-chain at site 3 (H-902) or 4 (H-903).

(5) Substitution of the pyridine ring's H atom at site 6 by a methyl group (H-901 *vs.* H-900) does not modify the effect of the compound.

(6) Substitution of a H atom of one of the amino groups of guanidine by a hydroxyphenyl group results in a more selective substance. The p-hydroxyphenyl derivative (V-138) is more selective than the m-hydroxyphenyl derivative (V-137).

(7) The o-chlorophenyl (V-130) and m-nitrophenyl (V-139) derivatives are of little effect.

(8) An aminophenyl group in para position (V-145) increases, in other position (V-140 and V-144) decreases, the effect of guanidine.

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Table II

Selective antiviral effect of guanidine and its 16 derivatives

Designation of derivative	Concentration causing ++ toxic effect	Smallest antiviral concentration, μg/ml, against		Effectivity index	
		PR8	Sendai	PR8	Sendai
Guanidine-HCl	3 000	500	500	6	6
H-900	3 000	75	75	40	40
H-902	4 000	750	750	5	5
H-903	16 000	1 500	1 500	10	10
H-901	1 750	75	37.5	23	46
H-904	4 000	50	75	80	50
H-540	500	37.5	37.5	13	13
H-539	750	75	75	10	10
V-53 (PYG)	4 000	125	50	32	80
H-915	12 000	1 500	1 500	8	8
V-130	3 000	1 000	n.t.	3	n.t.
V-137	750	75	75	10	10
V-138	750	50	37.5	15	20
V-139	1 000	> 500	n.t.	<2	n.t.
V-140	250	> 200	n.t.	<2	n.t.
V-144	4 000	1 500	n.t.	3	n.t.
V-145	2 000	200	200	10	10

n.t. = not tested.

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DISTRIBUTION IN THE ORGANS OF ^{131}I-OVALBUMIN ANTIGEN IN RAT AND MOUSE ANAPHYLAXIS

By

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Summary. (i) In rats with anaphylactic shock, the small intestine, lungs and heart contained high amounts of ^{131}I—OA, part of which was probably fixed to specific antibodies present in these organs. It has been concluded that these organs, especially the small intestine, are involved in the mechanism of rat anaphylaxis.

(ii) In mouse anaphylaxis the highest amount of ^{131}I—OA was demonstrated in the lungs, but radioactivity in the small intestine and skin also exceeded significantly the values for the control animals. Accordingly, in mouse anaphylaxis the lung has a definite role.

(iii) An association has been revealed between anaphylactic symptoms and localization of antibodies in the organs. It has been concluded that all anaphylactic symptoms can be explained by a disfunction of various organs impaired by the antigen-antibody reaction.

Injection of the eliciting antigen causes anaphylactic symptoms varying with the species of the sensitized animal. The difference in symptoms depends not only on the various mediator substances but also on the shock organs characteristic of the animal.

The severe morphological injuries in anaphylaxis are probably associated with the amount of antibodies fixed in the organ. For the determination of the so called "cytophilic" (sessile) antibodies responsible for anaphylaxis several methods have been elaborated. Of these the estimation of the amount of labelled antigens or in passive anaphylaxis the tracing and quantitative determination of labelled antibodies may be considered the most suitable techniques.

In anaphylactic shock the lungs of guinea pigs sensitized actively or passively contained 2 to 4 times higher amounts of labelled antigen than the lungs of the control animals [8, 15]. Intraportal eliciting injections of ^{51}Cr-ovalbumin resulted in a twofold increase of radioactivity in the liver, the shock organ of the dog [2]. Talmage *et al.* [13] and Dixon and Maurer [5] considered the elimination of labelled antibodies from the circulation of rabbits as an indicator of the degree of sensitization and of the antigen-antibody reaction *in vivo*. In mice, Masouredis *et al.* [9] and in completing experiments Melcher *et al.* [10] demonstrated the elimination from the circulation and fixation to the tissues and plasma of ^{131}I-antiovalbumin-rabbit globulin and ^{131}I-ovalbumin antigen and estimated the tissue levels for the antigen-antibody reaction. Radwan and West [12] showed that ^{131}I-labelled horse serum in-

jected into sensitized rats increased capillary permeability in the small intestine in such a degree that radioactivity could be demonstrated in the peritoneal cavity. In recent experiments [3] we have shown that in rat anaphylaxis high amounts of labelled ovalbumin appeared in the small intestine and the lung.

The purpose of the present investigations was to collect further data for the shock organs in rats and mice and to improve our knowledge of the mechanism of anaphylaxis.

Materials and methods

Sensitization and challenging. Wistar albino rats weighing 200—300 g were injected intraperitoneally at two day intervals with 3 doses of 60 mg crystalline ovalbumin (OA) dissolved in 1 ml saline. Together with the first antigen dose the animals received intraperitoneally *Bordetella pertussis* vaccine (BPV) comprising 5×10^9 cells. A similar dose of BPV was given to each animal intramuscularly 4 days before eliciting anaphylaxis. On the 12th day 60 mg ^{131}I—OA were injected intravenously as the eliciting dose. Fatal anaphylactic shock developed in 80—90% of the control animals.

Albino mice of both sexes weighing 15—20 g were sensitized intraperitoneally with 3 doses of 30 mg crystalline OA at two day intervals. In order to enhance the effect of sensitization, each mouse was given 2 doses of BPV adjuvant with 2×10^9 cells. The shock was elicited by the intravenous injection of 30 mg ^{131}I—OA in 0.5 ml volume. Eighty per cent of the control mice developed fatal anaphylaxis.

The control animals received without pre-treatment 60 mg (rats) or 30 mg (mice) ^{131}I—OA intravenously.

Determination of the antigen distribution. Immediately before death (between 30 and 60 minutes after the eliciting injection) the animals were bled by decapitation. The control rats and mice were sacrificed 30 minutes after the intravenous injection of ^{131}I—OA. Blood, liver, lung, small intestine, heart, spleen and abdominal skin specimens were placed into centrifuge tubes in 1 ml or 1 g amounts (rats) or in 0.1 ml or 0.1 g amounts (mice). Radioactivity was measured by the use of a well-type scintillation counter supplied with a NaI(Tl)-Bohrloch crystal. Then the haemoglobin content of the specimens was determined and the radioactivity for the blood remaining in the organs was subtracted from the total counts [7]. Data show activities corrected in this manner in cpm/g or in percentage of the control values.

The distribution of ^{131}I—OA was determined in 10 control and 10 test rats and in 15 control and 23 test mice. Statistical significance was tested by STUDENT's *t* method.

Results

In rats sensitized with OA and challenged with ^{131}I—OA a marked decrease in antigen activity was observed in liver and spleen. In blood and skin the levels were practically unaltered. In the small intestine, lung and heart, the activity increased significantly (Fig. 1A).

In Fig. 1B the values are expressed in percentage of the control. It is seen that the small intestine and the lungs had fixed more than double the amount of antigen fixed by the control preparations.

As the values were corrected for the radioactivity of residual blood, they indicated, at least partly, the amount of antigen fixed to antibodies in the corresponding organs. Accordingly, the most intensive antigen-antibody reaction occurs in the small intestine. As the amount of antigen fixed in the lungs

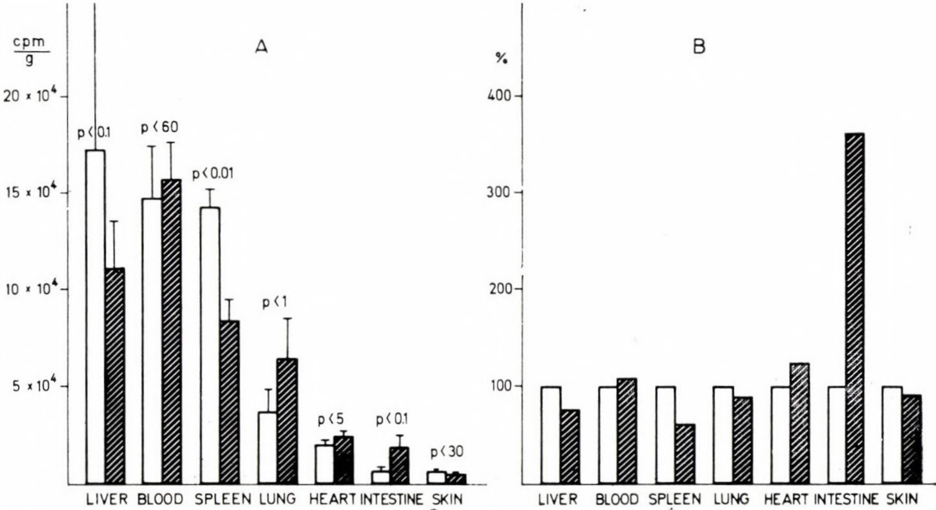

Fig. 1. Distribution of $^{131}I-OA$ in rats with anaphylactic shock. A = specific activity in various organs; B = $^{131}I-OA$ activity in various organs in percentage of control

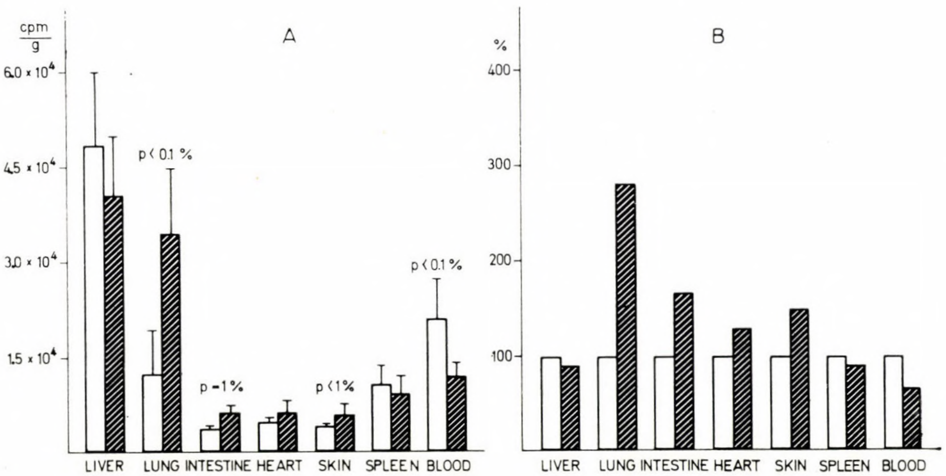

Fig. 2. Distribution of $^{131}I-OA$ in mice with anaphylactic shock. A = specific activity in various organs; B = $^{131}I-OA$ activity in various organs in percentage of control

and heart is considerable, these organs should also be considered to play a role in rat anaphylaxis.

In subsequent experiments anaphylaxis was elicited with $^{131}I-OA$ in mice sensitized with OA. As in the rat experiments, the antigen activity in the blood remaining in the organs was subtracted from the total activity.

Antigen activity in the circulation decreased markedly in the course of anaphylactic shock. A minimal decrease was observed in the spleen and the liver. In the lungs, the skin and the small intestine the activity increased significantly (Fig. 2A).

As indicated by the percentage comparison, the lungs fixed about three times, the small intestine and the skin about one and half times more antigen than did the corresponding organs of the control animals (Fig. 2B). As in any organ the amount of antigen is an indicator of the amount of specific antibodies, it may be stated that in mouse anaphylaxis the lungs play the most important part, but the small intestine and the skin are also involved in the mechanism.

Discussion

Our studies on the distribution of the eliciting antigen indicate that in the mechanism of anaphylaxis in the rat mainly the small intestine and, to a smaller extent, the lungs and the heart are involved. The severe functional and organic lesions observed are due to the antigen-antibody reaction taking place in the organs. The findings explain the observation of DAWSON, STARR and WEST [4] that haemoconcentration and haemorrhages are present in the heart and small intestine and sometimes in the lungs of animals dying 20 minutes after challenge. The explanation of the severe haemorrhages in the small intestine and of the dyspnoea marked by slow and irregular respiratory movements is also evident from our results. The progressive circulatory collapse and hypothermia are caused by various mediators (histamine, 5-hydroxytryptamine, bradykinin) released from the organs. The heart failure aggravates the systematic circulatory insufficiency.

Anaphylactic shock in mice is characterized by progressive circulatory insufficiency, cyanosis and oedema [11]. HARRIS and FULTON [6] and BERGMAN and MUNOZ [1] explained the above symptoms of tissue hypoxia by a failure of the peripheral circulation due to an effusion of plasma from the circulation into the tissues. The present experiments indicate that in mouse anaphylaxis the greatest accumulation of antigen occurs in the lungs, but activity in small intestine and skin is also significantly higher than in the respective organs of the control animals. As it is justified to state that antigen accumulation is due to the presence of specific antibodies in the organs, it may be concluded that the lungs play a cardinal role in the anaphylactic reaction. The shock mediators released in consequence of a functional injury of the lungs rapidly cause tissue hypoxia, a change responsible for all the other symptoms. Haemorrhage in the small intestine and oedema in the skin aggravate the circulatory failure and finally the animal succumbs to anaphylactic shock.

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SOME PROPERTIES OF ALKALINE PHOSPHATASE IN BACILLUS SPECIES

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Summary. *B. anthracis, B. cereus, B. megaterium* and *B. subtilis* contained repressible phosphatase. *B. subtilis* phosphatase was repressed at the lowest and *B. cereus* phosphatase at the highest phosphate concentration in the medium.

The four strains showed small differences in substrate specificity for para-nitrophenyl phosphate, beta-glycerophosphate, adenylic acid, glucose-1-phosphate, glucose-6-phosphate and pyrophosphate hydrolysis.

The Michaelis constant for the strains ranged between 2.8×10^{-5} and 4.1×10^{-5} *M*.

The sensitivity of phosphatase to complex-forming substances and heat showed differences characteristic of the strains.

The widespread occurrence of alkaline phosphatase in mammalian tissues and microorganisms is well-known [1, 2]. The properties of phosphatase have extensively been investigated in *Escherichia coli* [3, 4], *Bacillus subtilis* [5, 6], *Serratia marcescens* [7], *Pseudomonas fluorescens* [8], *Neurospora crassa* and in some yeasts [10]. Phosphatases in these microorganisms are similar in many respects such as alkaline pH optimum, moderate substrate specificity, divalent cation requirement for acitivity, and competitive inhibition by inorganic phosphate [2]. In other properties, *e.g.* molecular weight, amino acid composition, Michaelis constant, etc., the phosphatases may be different [6, 8, 11, 12].

The properties of phosphatase in the genus *Bacillus* have been examined only for *B. subtilis*. There are more data for the repression of alkaline phosphatase synthesis in some *Bacillus* species [13—16]. In the present study we have therefore compared the enzymologic properties of *B. anthracis, B. cereus, B. megaterium* and *B. subtilis* alkaline phosphatase in addition to studying their repressibility.

Materials and methods

Strains. B. anthracis Vollum (non-capsulogenic mutant), *B. cereus* W, *B. megaterium* 899 and *B. subtilis* Marburg were used. The cultures were maintained as spore suspensions.

Cultivation. LA medium [15] adjusted with 0.02 *M* K_2HPO_4 to 0.3 *mM* phosphate concentration was used. This phosphate concentration ensured suboptimal growth but maximum alkaline phosphatase activity for all strains.

The medium was seeded with 10^3 spores per ml, then incubated by aeration on a Gyrotory shaker (240 rev/min) for 16 to 18 hours at 37 °C. At the end of the incubation the cultures were in the early stationary phase.

Enzyme extracts. The culture was centrifuged at 4 °C then washed twice in 0.05 *M*, pH 8.5 Tris-HCl buffer. After washing the cells were resuspended to 1/20 of the original volume in the same buffer and treated in a MSE ultrasonic power unit at 0 °C. The cellular debris was then sedimented at 4000 g at 4 °C for 30 minutes and the supernatant was used as crude extract throughout the experiments.

Enzyme assay. To 2 ml appropriately diluted crude extract 2 ml reagent (0.5% para-nitrophenyl phosphate dissolved in 0.05 *M*, pH 8.5 Tris-HCl buffer) was added. After incubation at 37 °C for 10 minutes the reaction was terminated with 1 ml 2 *N* NaOH. The para-nitrophenol released was determined by the use of a Spectromom 201 spectrophotometer at 410 mμ. Enzyme activity was expressed in units, 1 unit corresponding to the amount of enzyme releasing 1 $m\mu M$ para-nitrophenol in 1 minute under the above conditions. Specific enzyme activity was expressed as 1 unit enzyme per 1 mg protein. Protein determination was performed by the colorimetric method of LOWRY *et al.* [17].

The method for estimating the substrated specificity of alkaline phosphatase differed from the above procedure in the use of Tris-HCl buffer containing 5 *mM* amounts of various phosphate esters and in determining inorganic phosphate after stopping the reaction by the method of LOWRY and LOPEZ [18].

Alkaline phosphatase repression was examined as described previously [15]. By adding 0.02 *M* solution to LA medium, a series of media containing 0.3, 0.5, 0.7, 0.9 and 1.1 *mM* inorganic phosphate was prepared. After 16 hours cultivation crude extracts were prepared from the cultures and their specific activities were plotted on a graph by considering the enzyme activity of the culture grown at 0.3 *mM* phosphate concentration as 100%.

Results

Repression conditions for alkaline phosphatase synthesis in *B. anthracis, B. cereus, B. megaterium* and *B. subtilis,* substrate specificity and affinity of phosphatases, inhibitory activity of phosphate, sensitivity to some complex-forming substances and heat inactivation of the phosphatases were compared.

Crude extracts prepared from the four *Bacillus* species showed different alkaline phosphatase activities. After cultivation, 90 to 95% of the total phosphatase activity were sedimented with the cells on centrifugation at 4000 *g* for 20 minutes. In repeated experiments the specific sedimented activity varied between 9.8—13.5 units/mg for *B. anthracis*, 26.5—32.8 units/ml for *B. cereus*, 11.7—16.2 units/mg for *B. megaterium* and 18.2—23.3 units/mg for *B. subtilis*.

Fig. 1 indicates the relative phosphatase activity of the cultures plotted against the inorganic phosphate content of the medium. Increasing of the phosphate ion content of the medium caused a decrease in alkaline phosphatase production by all strains examined. *B. subtilis* phosphatase was repressed at the lowest and *B. cereus* phosphatase at the highest phosphate concentration. The repression curves for *B. anthracis* and *B. megaterium* were similar and occupied an intermediary position between the curves for the former organisms.

Alkaline phosphatase is known to be slightly specific [2]; it catalyses the hydrolysis of a wide variety of compounds bearing a terminal phosphate group. In Table 1 the substrate specificity of our four strains is presented. All strains hydrolysed para-nitrophenyl phosphate, beta-glycerophosphate and adenylic acid with a similar degree of activity. Alpha-glucose-1-phosphate and alpha-glucose-6-phosphate were less readily attacked. Of the examined sub-

strates the lowest activity was observed for pyrophosphate. Accordingly, in substrate specificity the strains displayed small differences.

In order to characterize the substrate affinity of phosphatases the Michaelis constant was determined as described by LINEWEAVER and BURK [19].

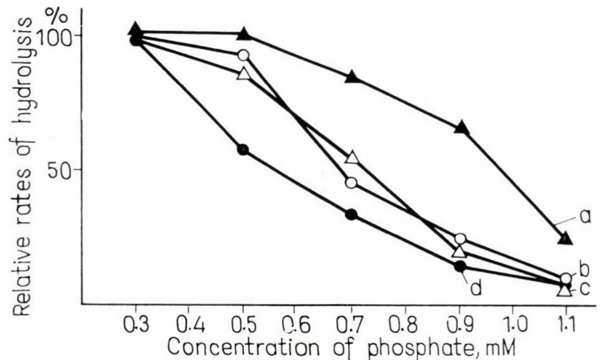

Fig. 1. Effect of phosphate concentration in the medium on alkaline phosphatase synthesis. a = *B. cereus;* b = *B. anthracis;* c = *B. megaterium;* d = *B. subtilis*

Table I

Substrate specificity of alkaline phosphatase in Bacillus species

Substrate	*B. anthracis*	*B. cereus*	*B. megaterium*	*B. subtilis*
para-Nitrophenyl-phosphate	100	100	100	100
beta-Glycerophosphate	107	102	94	112
Adenylic acid	89	93	100	98
Glucose-1-phosphate	52	65	60	48
Glucose-6-phosphate	64	54	48	57
Pyrophosphate	21	8	12	15

Enzyme activity was assayed by determining inorganic phosphate released from the substrate. Relative enzyme activity is expressed as percentage of the rate of para-nitrophenyl phosphate hydrolysis.

Graded amounts of para-nitrophenyl phosphate were dissolved in 0.05 *M* pH 8.5 Tris-HCl buffer and from the enzyme activities estimated at different substrate concentrations the K_m value was calculated. The Michaelis constant in *M* per litre was 2.8×10^{-5} for *B. anthracis,* 3.3×10^{-5} for *B. cereus,* 4.1×10^{-5} for *B. megaterium* and 3.9×10^{-5} for *B. subtilis.*

Alkaline phosphatase in *B. subtilis,* similarly as in other species, was effectively inhibited by inorganic phosphate [2]. The relative phosphatase

activities at various phosphate concentrations in the reaction system are presented in Table II.

Table II

Effect of phosphate concentration on alkaline phosphatase activity

Phosphate concentration, *mM*	*B. anthracis*	*B. cereus*	*B. megaterium*	*B. subtilis*
—	100	100	100	100
0.1	98	100	100	100
0.5	92	95	89	97
2.0	87	85	88	79
4.0	53	61	45	58
10.0	28	36	22	27

Relative phosphatase activity is expressed as percentage of enzyme activity in phosphate free system.

In the presence of 4.0 to 10.0 *mM* phosphate, enzyme activity decreased considerably in all strains. The cultures showed small differences in the degree of inhibition at various phosphate concentrations.

Table III indicates the sensitivity of the examined strains to complex-forming substances. These compounds bind divalent cations needed for the hydrolytic activity of the enzyme [2]. The four strains showed a similar degree of sensitivity to EDTA; *B. anthracis* phosphatase exhibited an increased resistance to trisodium citrate and *B. cereus* phosphatase to potassium cyanide.

Table III

Effect of complex-forming substances on alkaline phosphatase activity

Additions		*B. anthracis*	*B. cereus*	*B. megaterium*	*B. subtilis*
—		100	100	100	100
EDTA	10^{-4} *M*	100	93	95	100
	10^{-3} *M*	52	39	45	38
	10^{-2} *M*	0	5	0	0
Trisodium citrate	10^{-3} *M*	82	68	65	54
	5×10^{-3} *M*	74	37	26	31
	10^{-2} *M*	42	8	8	3
KCN	10^{-2} *M*	82	100	73	74
	10^{-1} *M*	23	87	14	7

Relative phosphatase activities at different concentration of complex-forming substances expressed as percentage of enzyme activity in the control system.

Heat sensitivity testing at 55 °C indicated that 50% of phosphatase in *B. anthracis, B. cereus* and *B. megaterium* were inactivated in 4 to 14 minutes, while *B. subtilis* phosphatase showed a decrease of only 8% even after 20 minutes exposure (Fig. 2).

At 70 °C *B. subtilis* phosphatase lost 50% of its activity in 4 minutes; the enzyme in the other strains was immediately inactivated at that temperature (Fig. 3).

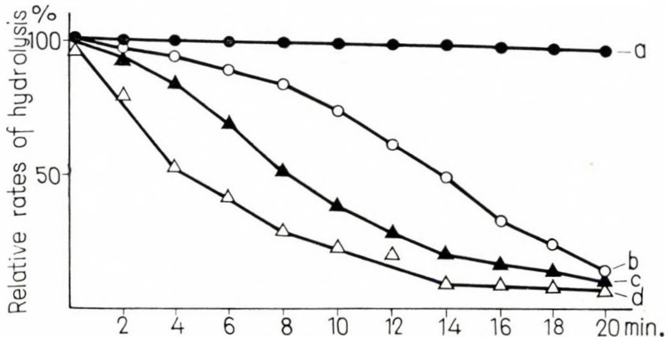

Fig. 2. Heat-inactivation of alkaline phosphatase at 55 °C. Abscissa: relative phosphatase activity in percentage of enzyme activity without heating. Ordinate: time of exposure. a = *B. subtilis;* b = *B. anthracis;* c = *B. cereus;* d = *B. megaterium*

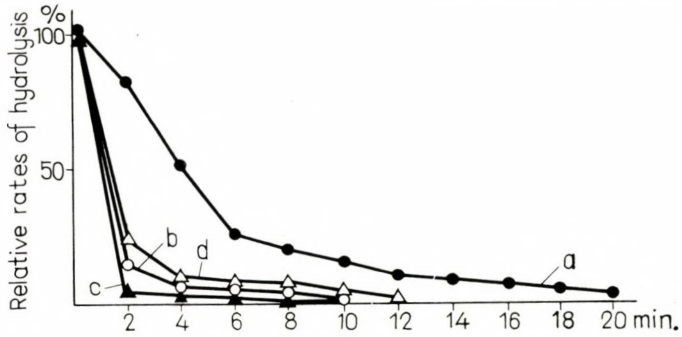

Fig. 3. Heat-inactivation of alkaline phosphatase at 70 °C. a = *B. subtilis;* b = *B. anthracis;* c = *B. cereus;* d = *B. megaterium*

Discussion

Horiuchi *et al.* [20], Torriani [21] and Garen and Levinthal [22] described alkaline phosphatase in *E. coli* as a repressible enzyme. At low inorganic phosphate content the culture exerts a high, with an excess of phosphate, a low phosphatase activity. Other strains were shown to behave similarly: *B. anthracis* [14, 15], *B. cereus* [14] and *B. subtilis* [13, 16]. In the present study we observed phosphatase repression at excess phosphate concentrations in

four different *Bacillus* strains, but the optimal phosphate ion concentration varied from strain to strain.

In *E. coli,* phosphatase is localized between the cell wall and the cell membrane [23], and on centrifugation it sediments with the cells. Protoplasts, in contrast, release the enzyme into the medium [24]. CASHEL and FREESE [25] observed that during cultivation 90% of *B. subtilis* phosphatase are present in the medium and only 10% remain in the cells. In contrast, according to TAKEDA and TSUGITA [6], alkaline phosphatase in *B. subtilis* is strongly bound to the cell wall and cell membrane. In the present experiments we found that in our 4 strains 90% to 95% of enzyme activity sedimented with the cells after culturing for 16 hours. This finding indicates that our strains are not essentially different in the localization of phosphatase.

As regards the Michaelis constant, substrate specificity and inhibitory effect of inorganic phosphate offer some explanations for the nature of enzyme-substrate binding.

TAKEDA and TSUGITA [6] determined the K_m value for *B. subtilis* crystalline alkaline phosphatase acting upon para-nitrophenyl phosphate as 3.6×10^{-5} *M* per litre. Our experiments revealed similar values not only for *B. subtilis* but also for the other 3 *Bacillus* species.

Within the experimental limits of error our strains were identical in substrate specificity. In repression by phosphate there were only small differences between the strains.

Accordingly, our *Bacillus* strains differed but slightly in substrate affinity, substrate specificity and repressibility. These findings show that the 4 strains were very similar in respect to enzyme-substrate binding.

For the function of alkaline phosphatase in *E. coli, B. subtilis* and other bacteria, divalent cations are needed [2, 6]. By binding these ions, complex-forming substances inhibit phosphatase activity. In our experiments the examined 3 substances exerted different effects. Trisodium citrate and potassium cyanide inhibited *B. anthracis* and *B. cereus* phosphatase less definitely than did the enzymes of the other strains. EDTA, in contrast, exerted a similar inhibitory activity on the phosphatase of all strains.

HEPPEL *et al.* [3] showed that *E. coli* phosphatase was highly heat-stable: in the presence of 10^{-2} *M* Mg^{++} it was resistant to 10 minutes exposure at 90 °C. As to our cultures, phosphatase was rapidly inactivated at 55 °C in *B. anthracis, B. cereus* and *B. megaterium,* but in *B. subtilis* it was destroyed only at 70 °C.

The different sensitivity to complex-forming substances and the high heat resistance of *B. subtilis* phosphatase indicate that, although the 4 enzymes are similar in enzyme-substrate binding, they differ in other properties.

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HEART GLYCOSIDES IN POLIOVIRUS HOST CELL INTERACTION

I. EFFECT OF DIGOXIN AND DIGITOXIN AND THEIR AGLUCONS ON ONE STEP GROWTH CURVES

By

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Summary. In a system consisting of type 1 (Mahoney) poliovirus and PMK III/1 permanent monkey cell line in Hanks' balanced salt solution with glucose, considerable reduction of final virion yield and an apparent prolongation of the lag phase took place in the presence of 10^{-6} or 10^{-7} M/l final concentrations of digoxin or digitoxin or their aglucons. In the concentration range from 10^{-8} to 10^{-11} M/l, both glycosides induced a concentration-dependent increase of the yield and shortening of the lag phase. Digoxigenin exerted a concentration-dependent inhibitory action in the 10^{-6} to 10^{-10} M/l concentration range. Two inhibition maxima and two minima were observed at 10^{-6} and 10^{-8} M/l and at 10^{-7} and 10^{-11} M/l concentrations, respectively. The inhibitory action of digitoxigenin tended to decrease in the range from 10^{-6} to 10^{-8} M/l, whereas no effect was produced at 10^{-9} M/l and lower concentrations.

Campbell and Kuechler [1] have reported ouabain to block the attachment of Mengo virus to L cells. Link *et al.* [2] observed inhibition of the cytopathic effect of Newcastle disease (ND) virus in rabbit lung fibroblast monolayer cultures on pretreatment with 2 mg/ml of lanatoside C or 25 μg/ml of ouabain. Both heart glycosides failed to affect viral cytopathogenicity when added to the system 24 hours after the infection. As far as we are informed no further or more detailed systematic studies have been performed along this line. Our earlier findings concerning the effect of saturated fatty acids [3] and some other surface active agents [4] on the virion host cell interaction suggested certain conformational changes of the cell membrane's building units to be of decisive importance. Heart glycosides known for their well-defined specific action on the activity and conformation of ATPase in the cell membrane were selected for further studies on the relations of membrane structure and virion—cell interaction.

Materials and methods

Cells and virus. The PMK III/1 permanent monkey kidney cell line and the type 1 (Mahoney) poliovirus strain were the same as used in previous studies [5]. All virus titrations were performed by plaque assay.

One step growth curves. Virus was adsorbed to suspended cells in the smallest possible volume (10^7 cells/0.1 ml) at 1 input multiplicity. Adsorption was allowed to take place under constant stirring for 10 minutes at room temperature and for an additional 5 minutes in a 37 °C water bath. Unadsorbed virus was removed by two subsequent washings in Hanks' balanced salt solution with glucose (HBS). Cells from the final sediment were then distributed into 100 ml airtight, siliconed Erlenmeyer flasks containing 10 ml HBS in 5% CO_2 atmosphere

so as to make a final concentration of 10^5 cells/ml. The flasks were incubated under constant gentle rotation at 37 °C in a water bath. Incubation was started exactly 30 minutes after the commencement of adsorption. This time was regarded as the zero point of the one-step growth experiment. The curves were obtained by titrating the virus content of samples taken at appropriate time intervals. The test substances were added to the system at the times indicated in the individual experiments.

Chemicals. All heart glycosides used in this study were standardized, crystalline products of G. Richter Pharmaceutical Co. Ltd., Budapest. Stock solutions and dilutions were prepared in absolute ethanol. Final dilutions in aqueous media were made in a single 100-fold step.

Experimental

Direct tests on monolayers. PMK III/1 cells were grown in tubes in a medium containing 40 vol. HBS, 45 vol. Parker 199, 10 vol. calf serum and 5 vol. of 5% lactalbumin hydrolysate. Confluent monolayers were obtained in 3 days. At that time the growth medium was replaced by maintenance medium (95 vol. Parker 199, 2 vol. calf serum, 3 vol. 10% bovine albumin solution) to which appropriate dilutions of glycosides were added. With each dilution 5 tubes were prepared and reincubated at 37 °C. Readings were made daily for 3 days. Digoxin, digoxigenin, digitoxin and digitoxigenin were tested at final concentrations ranging from 10^{-5} to 10^{-11} *M*/1. None of the test substances produced microscopically visible lesions in 72 hours at concentrations of 10^{-7} *M*/1 or lower. All of them inhibited further growth and caused granulation of the cells in 24 hours when added at 10^{-5} or 10^{-6} *M*/1 concentration. A notable cell detachment occurred only with 10^{-5} *M*/1.

Exposure test. Cells suspended in HBS at 10^5/ml concentration were exposed to 10^{-6} *M*/1 or lower final concentrations in HBS of each test substance under conditions corresponding to those of a one-step cycle experiment. After various periods of exposure the cells were harvested from the respective flasks, washed and explanted into tubes at a 3.3×10^5 cell/tube rate, with complete growth medium added. The tube cultures were incubated at 37 °C in a slanted stationary position. Readings were made once daily for 3 days. Results are shown in Table I.

Exposure of cells to 10^{-6} *M* concentrations of the individual substances for 10 minutes did not affect their ability to form a confluent monolayer in 24 hours. Digitoxin and digoxigenin appeared to cause some impairment after 3 to 5 hours exposure which, however, seemed to disappear on further incubation. Digitoxigenin and digoxin caused an exposure-dependent increase of growth inhibition. Morphologically no conspicuous difference was observed between treated and untreated individual cells, except for some granulation appearing in those exposed to the drugs for 7 hours. Results obtained with higher dilutions have not been tabulated in view of the absence of effects on growth and morphology even after 7 hours of exposure.

Effect of heart glycosides on the virus. Aliquots of a virion suspension containing 1.45×10^8 PFU/ml in HBS were exposed to 10^{-6} *M*/l final dilutions of

Table I

Recultivation of cells exposed to 10^{-6} M/1 heart glycosides for various periods

Substance	Exposure period	Growth after			Number of crosses	
		24^h	48^h	72^h	Mean	Total
0	10 min.	++++	++++	++++	4	19.6
	1 hour	++++	++++	++++	4	
	3 hours	++++	++++	++++	4	
	5 hours	++++	++++	++++	4	
	7 hours	+++	++++	++++	3.6	
Digitoxin	10 min.	++++	++++	++++	4	18.6
	1 hour	++++	++++	++++	4	
	3 hours	++	+++	++++	3	
	5 hours	+++	++++	++++	3.6	
	7 hours	++++	++++	++++	4	
Digitoxigenin	10 min.	++++	++++	++++	4	18.6
	1 hour	++++	++++	++++	4	
	3 hours	++++	++++	++++	4	
	5 hours	+++	++++	++++	3.6	
	7 hours	++	+++	++++	3	
Digoxin	10 min.	+++	++++	++++	3.6	12.8
	1 hour	+++	++++	++++	3.6	
	3 hours	++	+++	++++	3	
	5 hours	+	+	++	1.3	
	7 hours	+	+	++	1.3	
Digoxigenin	10 min.	++++	++++	++++	4	17.2
	1 hour	+++	++++	++++	3.6	
	3 hours	++	+++	++++	3	
	5 hours	++	+++	++++	3	
	7 hours	+++	++++	++++	3.6	

Explanation:
+ Cells attached to the glass, some divisions seen.
++ Cells attached to the glass, islands of outgrowth seen.
+++ Numerous islands of outgrowth, but no confluent monolayer formed.
++++ Confluent monolayer.

the substances tested. Incubation was made at 37 °C for 2 hours. Untreated virions in HBS served as controls. At appropriate intervals samples were taken and immediately diluted in glycoside-free HBS. Virus dilutions of 10^{-5}, 10^{-6} and 10^{-7}, representing 10^{-11}, 10^{-12} and 10^{-13} *M*/1 concentrations of the test substance were used for plaque count determinations on PMK III/1 monolayers. By this method no measurable (more than $\pm 20\%$) change was detectable in the PFU/ml contents of the treated and untreated virion suspensions.

Effect of various concentrations of test substances on the final virion production. One step growth curves were plotted for various concentrations of the individual test substances. The substances were added at zero time of the cycle

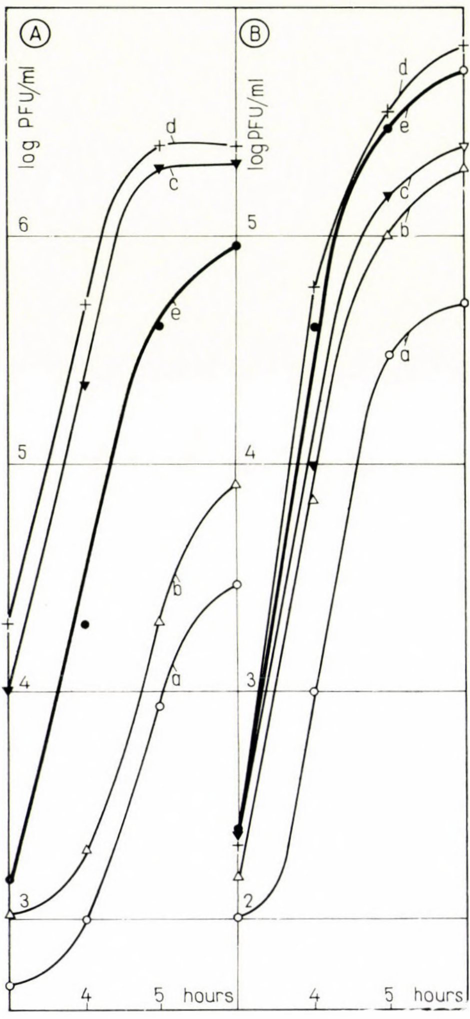

Fig. 1. Effect of digitoxin and digitoxigenin at various concentrations on the one step growth curve of type 1 poliovirus in PMK III/1 cells. A. Digitoxin. a: 10^{-6} *M*/1; b: 10^{-7} *M*/1; c: 10^{-8} *M*/1; d: 10^{-9} *M*/1; e: Control. B. Digitoxigenin. a: 10^{-6} *M*/1; b: 10^{-7} *M*/1; c: 10^{-8} *M*/1; d: 10^{-9} *M*/1; e: Control

experiment (see Methods) and were present throughout. Reference curves were those obtained in plain HBS. Results obtained with digitoxin and digitoxigenin are shown in Figs 1A and 1B.

Digitoxin at concentrations up to 10^{-7} $M/1$ appeared to reduce the virion yield by 90—99% and to prolong the lag phase. At 10^{-8} and 10^{-9} $M/1$ concentrations, a 2—3-fold increase of the yield and a reduction of the lag phase took

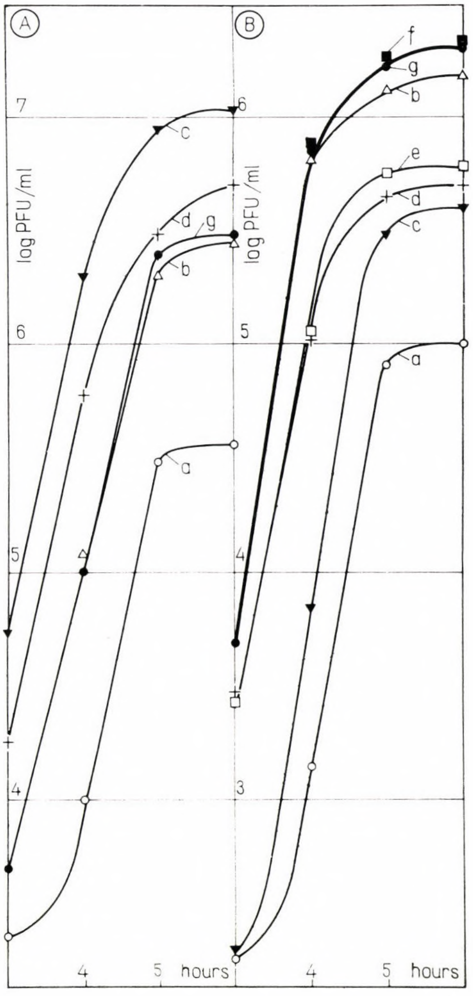

Fig. 2. Effect of digoxin and digoxigenin at various concentrations on the one step growth curve of type 1 poliovirus in PMK III/1 cells. A. Digoxin. a: 10^{-6} $M/1$; b: 10^{-7} $M/1$; c: 10^{-8} $M/1$; d: 10^{-9} $M/1$; g: Control. B. Digoxigenin. a: 10^{-6} $M/1$; b: 10^{-7} $M/1$; c: 10^{-8} $M/1$; d: 10^{-9} $M/1$; e: 10^{-10} $M/1$; f: 10^{-11} $M/1$; g: Control

place. Digitoxigenin at 10^{-6} $M/1$ concentration reduced the yield by about 90% and caused an apparent prolongation of the lag phase. Both effects tended to decrease on further diluting the substance. Interestingly, however, there

was no significant difference in the digitoxigenin effect at 10^{-7} and 10^{-8} $M/1$ concentrations, as consistently observed in repeated experiments. At 10^{-9} $M/1$ or lower concentrations, the drug failed to cause any change in the viral cycle curve.

Growth curves obtained in the presence of digoxin and digoxigenin at various concentrations are presented in Figs 2A and 2B.

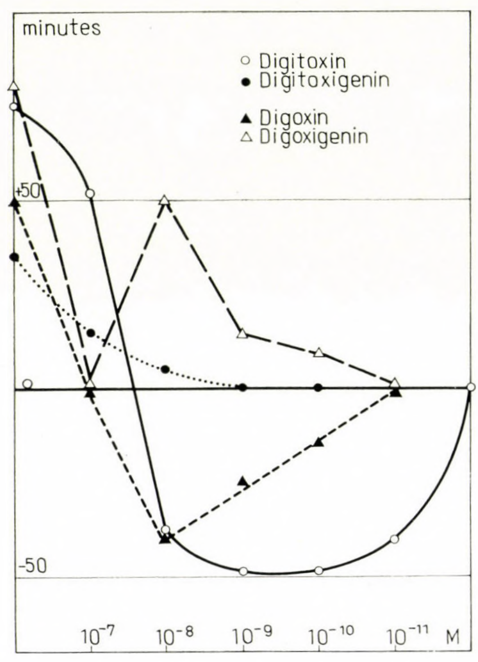

Fig. 3. Effect of glycosides and aglucons on the lag phase

Digoxin at 10^{-6} $M/1$ concentration reduced the virion yield by nearly 1 log unit and caused a prolongation of the lag phase. Both effects practically disappeared in the presence of digoxin at 10^{-7} $M/1$ concentration. In 10^{-8} $M/1$ dilution, the substance brought about a marked increase of yield and a reduction of the lag phase. At concentrations of 10^{-9} $M/1$ or lower, not shown in the graph, both effects tended to decrease. Digoxigenin reduced the yield by 99% at 10^{-5} $M/1$ concentration and by 95% at 10^{-6} $M/1$ concentration. In both cases the lag phase exhibited marked apparent prolongation. Digoxin at 10^{-7} and 10^{-11} $M/1$ concentration practically failed to affect the viral cycle's normal course. A marked concentration-dependent apparent increase of the lag and reduction of the yield was observed at dilutions of 10^{-8}, 10^{-9} and 10^{-10} $M/1$.

Effects on the temporal course of the viral cycle. The curves presented in Fig. 3 show the changes in the cycle's temporal course as effected by various

concentrations of the test substances. Points of the curves were obtained by arbitrarily extrapolating the straight parts of the individual cycle curves to a time axis (abscissa) drawn from the intersection of the growth curve in HBS and the virion concentration axis (ordinate) as zero point. In Fig. 3 the ordinate represents the apparent prolongation in plus minutes and the "shortening" in minus minutes, relative to the lag phase in the system in HBS (zero).

The effect of digitoxin on the temporal course was remarkable, whereas digitoxigenin caused a moderate prolongation which tended to decrease rapidly with the increase of the dilution. Digitoxin at 10^{-6} or 10^{-7} $M/1$ final concentration induced a considerable prolongation of the lag. This effect not only disappeared completely at the next 10-fold dilution step, but also changed into a reverse phenomenon. The lag reducing effect remained practically at the same level within the concentration range of 10^{-8} to 10^{-11} $M/1$.

Digoxin at 10^{-6} $M/1$ concentration prolonged the lag phase by about 50 minutes. At 10^{-7} $M/1$ neither prolongation nor a shortening of the lag was observed, whereas at 10^{-8} $M/1$ the drug reduced the lag phase by about 40 minutes. With increasing dilution, the lag reducing effect tended to decrease until it disappeared at a concentration of 10^{-11} $M/1$. The curve representing the effects of various digoxigenin concentrations on the lag phase shows two prolongation maxima. The first was at 10^{-6} $M/1$ and the second at 10^{-8} $M/1$, whereas at 10^{-7} $M/1$ the lag phase was unaffected. At dilutions higher than 10^{-8} $M/1$, the prolonging effect tended to decrease rapidly. Of the digoxigenin concentrations tested, none had reduced the lag phase.

Discussion

Digitalis derivatives at the concentrations tested had no notable influence on either the virions or the cells, but when added at zero time to suspended cells carrying adsorbed virions, the same compounds considerably affected the viral cycle's further course. Digitoxin and digoxin were either stimulatory or inhibitory depending on their actual concentration. Digitoxigenin and digoxigenin were either inhibitory or inactive throughout the concentration ranges tested. The abrupt reduction of the inhibitory effect of digoxigenin at 10^{-7} $M/1$ as well as the reappearance of the phenomenon at lower concentrations was remarkable, though hard to explain.

Earlier studies in this laboratory on the effect on the viral cycle of certain saturated fatty acids and surfactants [3, 4] have shown that these substances exerted their stimulatory or inhibitory actions within relatively narrow ranges of critical concentrations.

High concentrations of digitalis glycosides (10^{-6}—10^{-7} $M/1$) were required to reduce the final virion yield by 90—99%. Calculating the mean minimal

spherical surface area of a PMK III/1 cell from diameter measurements on electron microscopic pictures of suspended cells, a value of 1×10^{11} Å^2 (radius = 10 μ) was obtained. Assuming that the adsorption of glycosides was complete and irreversible, and taking Avogadro's number (6×10^{23}) into consideration, the number of glycoside molecules per cell surface area was one molecule per 16 and 160 Å^2 for 10^{-6} and $10^{-7}M/1$ concentrations, respectively. At a molecular density of this order, the coating of cells seems to be highly probable. Data presented by others have in fact shown that pretreatment of cells with heart glycosides at such concentrations resulted in an extensive inhibition of virion adsorption [1]. In the present experiments, the virions were already adsorbed to the cells, but certainly only part of them was attached irreversibly. In this context, we refer to our view that the irreversible attachment is in fact a firm binding of 3 vertices on one and the same virion facet to 3 complementary receptor sites on the cell membrane [4]. The process of irreversible attachment is supposed to involve an orderly sequence of short range (van der Waals type) interactions consecutive to the adsorption by long range (Coulombic) forces. Let us suppose that at zero time of the cycle, part of the virions is only adsorbed, part of them partially attached, while a very small fraction is irreversibly attached by all the 3 vertices required. Coating of such cells by glycoside will certainly not only interfere with the further progress of irreversible virion attachment, but also hinder the energy requiring phase of eclipse. Thus at high concentrations the inhibitors appeared to have at least two points of attack.

Clearly, this type of explanation is not valid for the effects observed at lower concentrations of the glycosides, where the density of adsorbed glycoside molecules was one per each 1600, 16 000 or 160 000 Å^2 of the cell surface area. Yet we believe that the lower concentrations should be used for the detailed analysis of the mechanism of the stimulatory or inhibitory actions of digitalis derivatives. Further studies along this line are in progress.

Acknowledgement. The valuable technical assistance of Miss EDITH TAKÁCS is gratefully acknowledged.

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HEART GLYCOSIDES IN POLIOVIRUS HOST CELL INTERACTION

II. EFFECT OF THE TIME OF ADDITION ON STIMULATORY OR INHIBITORY ACTION

By

EMESE GYÖRGY and A. KOCH

National Institute of Public Health (Director: T. BAKÁCS), *Budapest*

(Received March 10, 1969)

Summary. Digitoxin, digoxin and their aglucons at 10^{-6} *M*/1 or 10^{-8} to 10^{-9} *M*/1 concentrations were added to suspended PMK III/1 cells at different points of time following the adsorption of type 1 (Mahoney) poliovirus. The effect was detected by plotting one step viral cycle curves. The time of addition was considerably affecting the efficiency of both inhibition and stimulation. Maximum effect was consistently observed if the addition had been made at zero time. Low concentrations of the substances proved inefficient if added after one hour or later.

At high concentrations the later the addition had taken place the weaker was the inhibitory activity. With digitoxigenin, digoxin and digoxigenin, a sudden decrease of the effect occurred between the first and second hour. Apparently, digitalis derivatives interfered with the irreversible attachment and/or with some early step of the uptake (eclipse) of absorbed virions.

Depending on their concentration, heart glycosides have been shown to reduce or to increase the final virion yield. Aglucons were either inhibitory or inactive under similar conditions [1]. To identify the digitalis sensitive phase of host cell virion interaction, we have studied the influence of the time of addition of the active substances at different concentrations on the course of the viral cycle.

Materials and methods

The virus, cells, and methods used have been described previously [1].

Experimental

Type 1 (Mahoney) poliovirus was adsorbed to cells of PMK III/1 permanent monkey kidney line at 1 input multiplicity. The suspensions of cells with adsorbed virions were incubated in a 37 °C water bath under constant gentle rotation. The substances were added at different concentrations at 0, 1, 2, or 3 hours time of the viral cycle. Samples for virion content (PFU/ml) determination were taken at predetermined intervals to obtain cycle curves. Controls in Hanks' balanced salt solution with glucose (HBS) were set up with each test. For further details of the procedure, an earlier paper should be consulted [2].

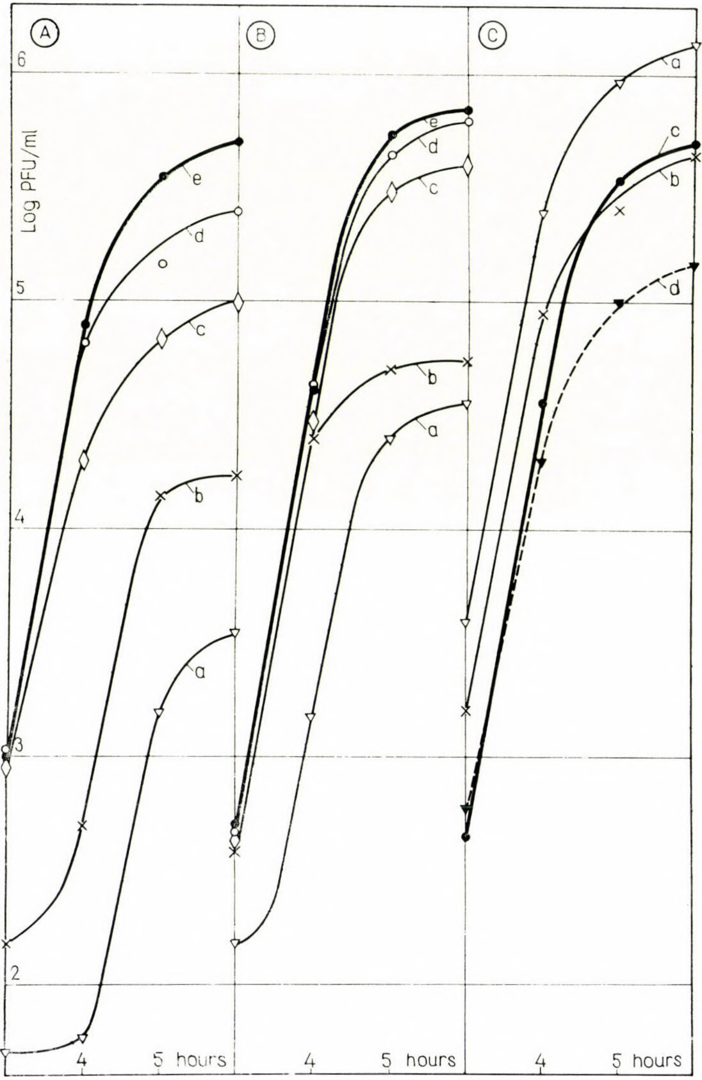

Fig. 1. Effect of the time of addition on the yield reduction action. A. Digitoxin, 10^{-6} M/l. Time of drug addition, a: 0; b: 1 hour; c: 2 hours; d: 3 hours; e: Control. B. Digitoxigenin, 10^{-6} M/l. Time of drug addition, a: 0; b: 1 hour; c: 2 hours; d: 3 hours; e: Control. C. Digitoxin, 10^{-8} M/l. Time of drug addition, a: 0; b: 1 hour; c: Control. Digitoxigenin, 10^{-8} M/l. Time of drug addition, d: 0

Representative sets of curves obtained with digitoxin and digitoxigenin are shown in Figs 1A, 1B, and 1C; those obtained with digoxin and digoxigenin, in Figs 2A, 2B and 2C.

For detailed analysis of results, final yields were plotted against the times

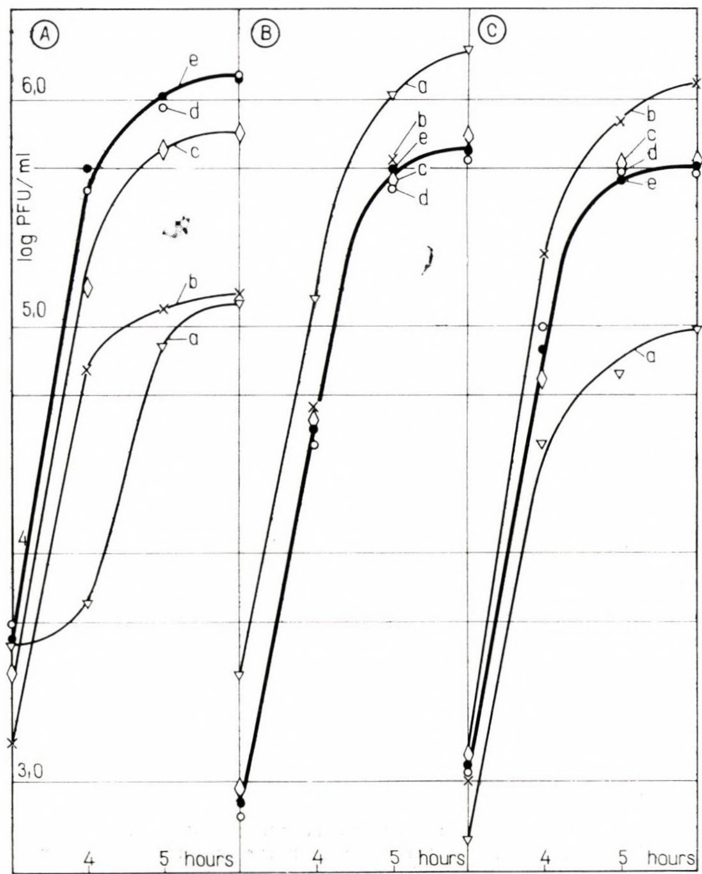

Fig. 2. Effect of the time of addition on the yield reducing action. A. Digoxin, 10^{-6} *M*/l. Time of drug addition, a: 0; b: 1 hour; c: 2 hours; d: 3 hours; e: Control. B. Digoxin, 10^{-8} *M*/l. Time of drug addition, a: 0; b: 1 hour; c: 2 hours; d: 3 hours; e: Control. C. Digoxigenin, 10^{-}. *M*/l. Time of drug addition, a: 0; b: 1 hour; c: 2 hours; d: 3 hours; e: Control

of addition, referred to the control in HBS as unit. A curve obtained in earlier studies [3] using arachidic acid has also been included for comparison.

Digitoxin and digitoxigenin (Fig. 3A) were examined at concentrations of 10^{-6} *M*/l, 10^{-8} or 10^{-9} *M*/l. Analysis of the 10^{-6} *M*/l digitoxin curves showed a steady decrease of the inhibitory action with the time of addition. The decrease was 5.66-fold from 0 to the first hour, 5.9-fold from the first to the second hour and 2.50-fold from the second to the third hour. The total decrease in inhibitory activity was about 33-fold in the period from 0 to the second hour, *i.e.*, 13.2 times more than in the period from the second to third hour. Digitoxigenin at the same concentration caused only a 1.4-fold decrease of inhibitory action up to the first hour; an about 8.5-fold decrease occurred from the first

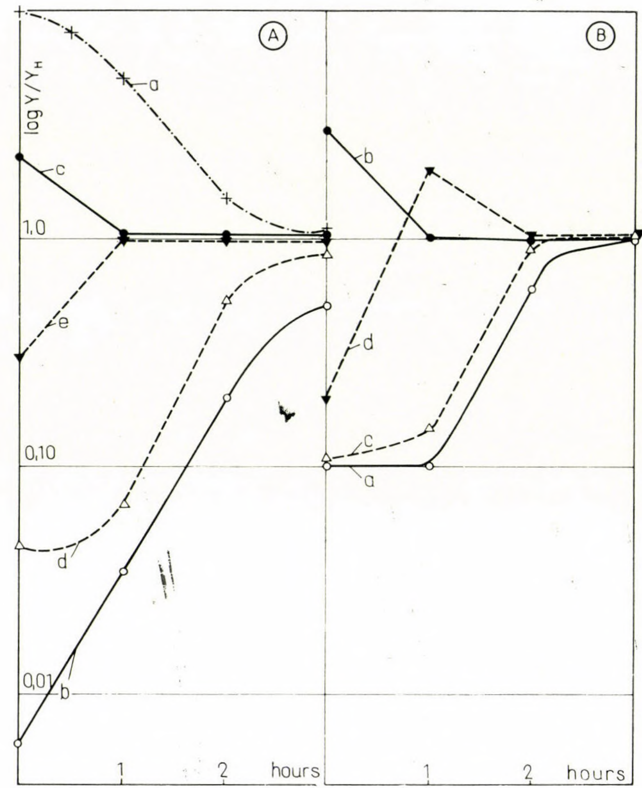

Fig. 3. Relative yield reducing action of substances added at different concentrations at different points of time. log Y/Y_H: ratio of yield in the presence (Y) and in the absence (Y_H) of active substance. The yield in HBS (Y_H) was taken as unit. Hours: hours elapsed before drug addition A. a: arachidic acid 10^{-5} *M*/1; b: digitoxin 10^{-6} *M*/1; c: digitoxin 10^{-8} *M*/1; d: digitoxigenin 10^{-6} *M*/1; e: digitoxigenin 10^{-8} *M*/1. B. a: digoxin 10^{-6} *M*/1; b: digoxin 10^{-8} *M*/1; c: digoxigenin 10^{-6} *M*/1; d: digoxigenin 10^{-8} *M*/1

to the second hour and up to the third hour the decrease was only 1.5-fold in contrast to about 12-fold during the first two hours. At low concentrations, digitoxin exhibited a stimulatory, while digitoxigenin an inhibitory, action exclusively when added at 0 time.

The results obtained with digoxin and digoxigenin at various concentrations are shown in Fig. 3B. At 10^{-6} *M*/1 concentration, the inhibitory effect of both digoxin and digoxigenin was found to decrease abruptly in the interval between the first and second hours of the cycle. 10^{-8} *M*/1 of digoxigenin was inhibitory when added at 0 time, but increased 2-fold the final yield when added at the end of the first hour of the cycle.

Discussion

Previous studies [1] of quantitative relations of the effect of digitalis derivatives on the viral cycle have been suggestive of two possible types of action.

(1) The presence of heart glycosides at high concentrations (10^{-6} and 10^{-7} $M/1$) seemed to inhibit the process of the virions' irreversible attachment through coating the total available cell surface (one molecule per each 16 or 160 $Å^2$ area) and to hinder the eclipse by inhibiting the membrane ATPase. Disturbance of certain cellular metabolic processes required for the production of a full virion yield seemed unlikely as in the absence of virus no conspicuous cellular damage was demonstrable after exposure to the substances at 10^{-6} $M/1$ concentration for 7 hours at 37 °C.

(2) At low concentrations, the digitalis derivatives cannot form a coherent coat, but will attach in a highly specific way to their receptor, the ion transfer ATPase enzyme. As shown by studies on purified enzyme preparations [4] digitalis derivatives at 10^{-8} to 10^{-10} $M/1$ concentration will generally induce a less than 10% inhibition of enzyme activity. Similar concentrations were, however, found to stimulate contractions of single cultured myocardial cells [5]. Thus, the effect on the viral cycle of digitalis concentrations of the given orders of magnitude are very likely caused by mechanisms other than coating of the cell surface and massively inhibiting the functioning of a transfer ATPase.

The present results revealed that whatever the mechanism of virion yield reduction, it manifests itself during the attachment and/or penetration period of the viral cycle. With low concentrations of digitalis derivatives, the inhibitory effect was limited to a phase accomplished between 0 and one hour. Within this period of time, irreversible attachment is certainly completed, while the eclipse has just started. The complete lack of effectiveness on addition at 1 hour makes a significant inhibition of the eclipse very improbable. Thus it seems that low concentrations will interfere chiefly with the process of irreversible attachment.

With higher concentrations, the inhibitory action was nearly the same at zero time and by the end of the first hour, but diminished significantly by the end of the second hour. This implies that high concentrations will interfere with a process not completed by the end of the first hour, but irreversibly accomplished soon afterwards. According to the general view, this process is the uptake (or eclipse) of the irreversibly attached virion. This phase requires energy, thus it is very likely sensitive to the massive inhibition of membrane ATPase. Thus, the inhibition of attachment and eclipse seem to be the main effects at zero and one hour, respectively. The persistence of an excess amount of the inhibitor added at high concentration at zero hour interfered further with

the penetration of those virions which had escaped the inhibition of irreversible attachment. When the inhibitor was added after one hour, part of the initially irreversibly attached virions were already in the eclipse phase and were no more accessible to the inhibitor. By the end of the second hour, attachment and eclipse had obviously been completed and the addition of glycosides appeared to lack any significant effect on the further course of the viral cycle. The persistence of a roughly 50% yield reducing activity of 10^{-6} $M/1$ of digitoxin on addition as late as the third hour appears to suggest the involvement of some general effect of this substance on cell metabolism under the given experimental conditions.

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EMESE GYÖRGY, A. SÁNDOR KOCH,
National Institute of Public Health, Gyáli út 2–6, Budapest IX, Hungary

INDEX

Tomus XVI

Fasciculus 2

Hollós, I.: Effect on Influenza Virus of a Modified Francis Inhibitor and Its Acetone-Soluble Fraction. III. Experiments on the Mouse Lung 113

Gadó, I., Boromissza, É., Horváth, I.: Induction and Multiplication of λ-Phage. III. The Effect of Hydroxyurea on Heat Induction 123

Novák, E. K., Darvas, E., Zsolt, J.: Breakdown of Oligosaccharides by Some Afermentative Yeast Species 131

Füst, G., Keresztes, M.: Effect of Endotoxin on the Serum Level of Complement Components. II. Effect of Endotoxin on Dog Serum Complement Level *in vivo* and *in vitro* 135

Novák, E. K., Kevei, F., Oláh, B., Zsolt, J.: Investigations on the Oligosaccharide Decomposition by *Endomycopsis wickerhamii* van der Walt 149

Ralovich, B., Emődy, L.: Observations on Primary Bacteriaemia 153

Rauss, K., Kétyi, I., Máté, J., Kneffel, P., Ambó, G., Maróczi, J., Pusztai, S., Joó, I., Szendrei, L.: New Observations on Oral Vaccination against Dysentery 159

Váczi, L., Hankovszky, O., Hideg, K., Gergely, L., Hadházy, Gy.: Selective *in vitro* Antiviral Effect on Myxovirus Strains of Guanidine Derivatives 171

Csaba, B., Deseő, G., Kávai, M.: Distribution in the Organs of ^{131}I-Ovalbumin Antigen in Rat and Mouse Anaphylaxis 175

Dobozy, A., Hammer, H.: Some Properties of Alkaline Phosphatase in Bacillus Species 181

Koch, A., György, E.: Heart Glycosides in Poliovirus Host Cell Interaction. I. Effect of Digoxin and Digitoxin and Their Aglucons on One Step Growth Curves 189

György, E., Koch, A.: Heart Glycosides in Poliovirus Host Cell Interaction. II. Effect of the Time of Addition on Stimulatory or Inhibitory Action 197

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G. D. GIFFORD and G. G. PRITCHARD Toxicity of hyperbaric oxygen to yeasts displaying periodic enzyme synthesis

L. HANKIN and P. E. KOLATTUKUDY Degradation of ursolic acid, a major component of apple wax, by a pseudomonad isolated from soil

J. A. KANE and W. W. KARAKAWA Immunochemical analysis of *Streptococcus bovis*, strain S19, cell walls

A. B. LLOYD Behaviour of streptomycetes in soil

I. W. DAWES, D. KAY and J. MANDELSTAM Spoluration in *Bacillus subtilis.* Establishment of a time scale for the morphological events

D. R. BIGGINS and J. R. POSTGATE Nitrogen fixation by cultures and cell-free extracts of *Mycobacterium flavum* 301

G. H. G. DAVIS and K. G. NEWTON Numerical taxonomy of some named coryne form bacteria

P. WHITNEY, J. M. CHAPMAN and J. B. HEALE Carboxymethylcellulase production by *Verticillium albo-atrum*

T. IINO Polarity of flagellar growth in salmonella

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INHIBITION OF THE DEVELOPMENT OF PRIMARY ANTITOXIC IMMUNITY BY HETEROLOGOUS ANTILYMPHOCYTE SERUM*

By

L. Réthy and L. Hegedüs

Institute for Serobacteriological Production and Research "Human" (Director: G. Veres), Budapest

(Received February 7, 1969)

Summary. Mice were primed with graded doses of tetanus toxoid and subjected to toxin challenge test in order to demonstrate the effect of heterologous antilymphocyte serum (ALS) on the development of active immunity against tetanus.

(i) ALS effectively inhibited the development of primary immunity against tetanus.

(ii) Immunosuppression by ALS was effective only in the early stage of priming. Administration of ALS shortly before and shortly after immunization exerted the same effect.

(iii) In the productive stage of antitoxic immunity ALS was ineffective.

(iv) As to the mode of action of ALS our experiments confirmed theories based on the "blindfolding" phenomenon.

(v) Antigenic competition in the immunosuppressive effect of ALS could be excluded under the experimental conditions.

Antilymphocyte serum (ALS) is known to be a suitable agent for the inhibition of cell-mediated immune reactions. It prolongs the survival of allografts and inhibits the rejection of xenografts, abrogates the second-set reaction, *i.e.* the increased immune reaction after a repeated transplantation [1—5]. ALS inhibits the development of tuberculin-type "delayed" allergic reaction, or, probably by erasing immunological memory, decreases or eliminates the state of sensitivity established by the antigenic stimulus [6—8]; it may also suppress the production of humoral antibodies against heterologous erythrocytes, bacterial antigens or heterologous albumin [9—12].

The present studies were undertaken in view of the fact that the immunosuppressive effect of ALS has not satisfactorily been elucidated in immunological reactions based mainly on circulating antibodies. As a model, antitoxic immunity developing on priming with tetanus toxoid was chosen. The following problems were examined.

1. Effect on immunosuppression of ALS administration (a) before the antigenic stimulus; (b) within some days after the antigenic stimulus, *i.e.* in the inductive phase of immunity; (c) in the productive stage of antibody formation.

2. As ALS might act as a protein antigen, the role of antigenic competition [18, 19] in the immunosuppressive effect was also examined.

* Part of this study was performed during a W.H.O. fellowship in the National Institute for Medical Research, Mill Hill, London.

Materials and methods

1. *Test antigen.* One ml antigen contained 12.5 BU tetanus toxoid (purified with trichloroacetic acid; purity grade 900 BU/mg PN) adsorbed to aluminium phosphate representing 1 mg Al^{+++} [20, 21].

2. *Active immunization.* Random-bred Swiss mice were distributed into groups of 15 and were injected with graded doses of tetanus toxoid: twofold serial dilutions were administered in single doses of 0.5 ml.

Toxin challenge test was performed 21 days after immunization: each mouse was injected with 20 LD_{50} of tetanus toxin [13].

3. *Checking of active immunity.* Animals subjected to toxin challenge were observed for 6 days. The number of dead animals was recorded daily. PD_{50} values (50% protective doses of tetanus toxoid) were estimated for each group from the proportion of survivals.

4. *Preparation of ALS.* The thymus of male mice weighing 9—10 g was removed after bleeding by decapitation. The homogenized material was suspended in Hanks' solution and the tissue debris was sedimented. Living thymocytes were adsorbed to aluminium phosphate adjuvant and used for the immunization of rabbits in two intramuscular doses at two-week intervals. Each dose represented 3×10^8 thymocytes per kg body weight. Seven days after the second dose the rabbits were bled. The sera were pooled and stored in the deep freezer without inactivation.

5. *Assay of the effect of ALS.* 5.1. *Lymphoagglutinating and lymphocytotoxic effects* were assayed *in vitro* after inactivation of ALS at 56 °C. For the cytotoxic test the system was supplemented with rabbit serum complement [14, 15].

5.2. *In vivo tests.* 5.2.1. *Graft-protectivity.* The tail-skin of inbred CBA female mice was transplanted to random-bred Swiss female mice weighing 18—20 g. The procedure was performed under pentobarbital anaesthesia as described by BILLINGHAM and MEDAWAR [16]. Each grafted mouse was injected with 0.5 ml ALS, 2 and 5 days after grafting. The grafts were fixed with plaster of paris for 9 days. After removal of bandages the condition of the grafts was checked daily. Medium survival time (MST) was calculated and used for characterizing the graft protective potency of ALS.

As controls, untreated grafted mice, and grafted mice injected with normal rabbit serum (NRS) were used.

5.2.2. *Immunosuppression of the effect of tetanus toxoid.* The schedule of the experiments is presented in Table I.

5.2.3. *Toxicity test.* Five doses of 0.5 ml ALS were injected into mice at two-day intervals. The animals were observed for 30 days. ALS was regarded as "non-toxic" if all animals survived and had lost not more than 20% of their weight or showed no symptoms indicating haemolysis.

6. *Evaluation of the results.* PD_{50} values were calculated for each group and compared to the control. In this manner the relative potency (RP) obtained under different experimental conditions was estimated.

Statistical significance was estimated by the *t* test. The 95% confidence limits were also calculated.

Table I

Schedule of the experiments

Groups of mice		Days before or after immunization							
Designation	Treatment	−5	−2	0	+2	+5	+16	+19	+21
1	—	—	—	priming	—	—	—	—	T
2	ALS +2 + 5	—	—	priming	ALS	ALS	—	—	T
3	ALS — 2— 5	ALS	ALS	priming	—	—	—	—	T
4	ALS+16+19	—	—	priming	—	—	ALS	ALS	T
5	NRS+2+5	—	—	priming	NRS	NRS	—	—	T
6	NRS—2—5	NRS	NRS	priming	—	—	—	—	T

ALS = antilymphocyte serum, 0.5 ml per dose
NRS = normal rabbit serum, 0.5 ml per dose
T = toxin challenge, 20 LD_{50} per mouse

Results

Results of *in vitro* tests with heterologous rabbit-anti-mouse antilymphocyte serum and with control normal rabbit serum are presented in Table II.

It is seen that ALS showed 1 : 128 lymphoagglutinating, 1 : 512 cytotoxic and 1 : 8 heterohaemagglutinin titre; the normal serum was inactive.

Table II

In vitro effect of heterologous antilymphocyte serum

Test	Result	
	ALS	NRS
Lymphoagglutination	1 : 128++	1 : 2±
Cytotoxicity	1 : 512++	1 : 2?
Mouse erythrocyte agglutination	1 : 8++	negative

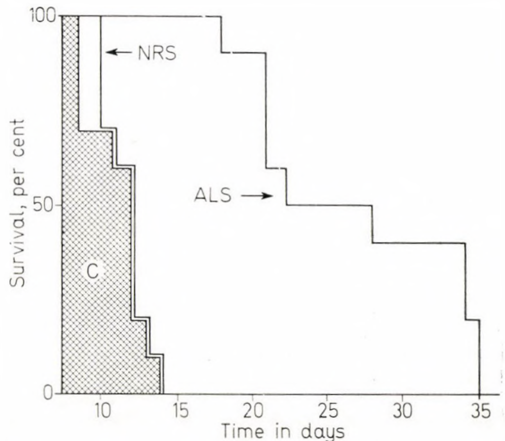

Fig. 1. Graft protective activity of heterologous antilymphocyte serum in mice. C = untreated controls, MST = 11.3 days; ALS = heterologous antilymphocyte serum-treated animals, MST = 26.1 days; NRS = normal rabbit serum, MST = 11.6 days. Two 0.5 ml doses of ALS and NRS were injected on the 2nd and 5th days after grafting

Toxicity test in mice indicated that neither ALS nor NRS was toxic.

The dynamics of the graft protective effect of ALS are shown in Fig. 1.

Fig. 1 shows that in dynamics of rejection almost identical results were obtained in untreated and in NRS-treated mice (MST = 11.3 and 11.6 days, respectively). Immunological rejection of the grafts began on the 9th or 10th day; none of the grafts survived for more than 14 days.

Rejection of grafts in ALS-treated mice began 16 days after grafting and was complete by the 35th day. The MST for ALS-protected grafts was 26.1

days. Immunological rejection of the grafts occurred proportionally before and after the point of time corresponding to the MST-value.

In immunization with adjuvanted tetanus toxoid the ALS characterized by the above data acted as shown in Fig. 2.

Column 1. No. The control group received only tetanus toxoid priming and was challenged with tetanus toxin after 21 days. It is seen that 0.075 m

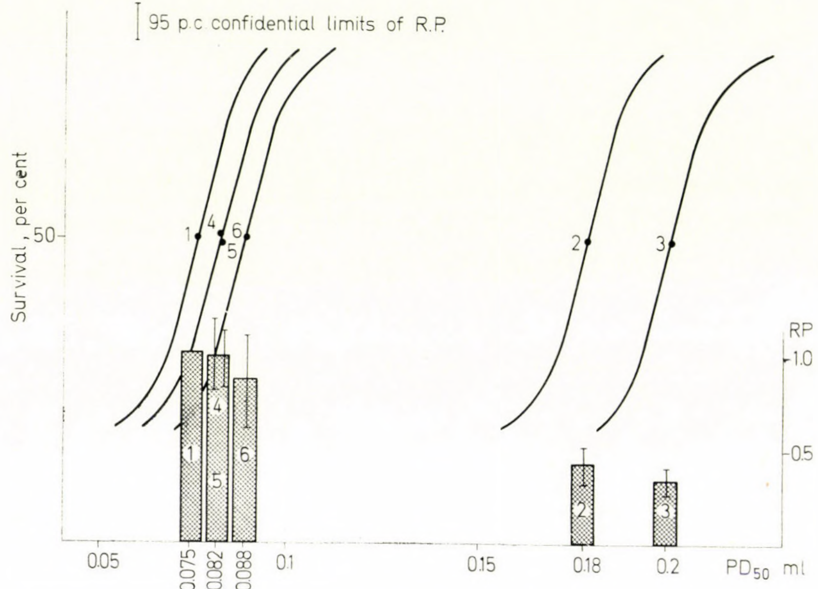

Fig. 2. Suppression of the primary immune effect of adsorbed tetanus toxoid with heterologous ALS. 1 = untreated control of active immunity to tetanus; 2 = ALS-treatment on days 2 and 5 following; 3 = ALS-treatment on days 5 and 2 prior to; 4 = ALS-treatment on days 16 and 19 following; 5 = NRS-treatment on days 2 and 5 following, and 6 = NRS-treatment on days 5 and 2 prior to, immunization with tetanus toxoid. Columns = degrees of relative potency-values as compared to the efficacy of untreated control

of tetanus toxoid protected 50% of the animals against challenge with 20 LD_{50} per mouse of tetanus toxin.

Column No. 2. If ALS was administered 2 and 5 days after immunization, the value of PD_{50} increased to 0.179 ml. Accordingly, the RP of tetanus toxoid in the test-group decreased to 0.42 in relation to the untreated control group.

Column No. 3. If ALS was given 5 and 2 days before immunization, PD_{50} was 0.2 ml, corresponding to a RP of 0.37.

Column No. 4. When ALS was injected 16 and 19 days after priming with tetanus toxoid (*i.e.* 2 and 5 days before the toxin-challenge) PD_{50} was 0.082 ml, corresponding to a RP of 0.98.

Columns No. 5 and 6. Control experiments performed in order to show the presence or absence of antigenic competition indicated that NRS did not influence the immunological effect of tetanus toxoid. In the group treated with NRS before immunization 0.85, in that treated after immuzation 0.98 RP values were demonstrated.

The statistical analysis of the differences is presented in Table III, where the degrees of significance are expressed in per cents.

Table III

Results of statistical analysis in per cent

	Control	ALS +2+5	ALS −2−5	ALS +16+19	NRS +2+5
ALS+2+5	0.1				
ALS−2−5	0.01	~5.0			
ALS+16+19	homog.	0.2	0.1		
NRS+2+5	homog.	0.1	0.01	homog.	
NRS−2−5	>5.0	0.1	0.01	>5.0	homog.

Comparison of the results shown in Fig. 2 and Table III allowed the following conclusions.

1. A statistically significant decrease in the immunological effectiveness of tetanus toxoid was demonstrated only when ALS treatment had been performed shortly before or after priming. ALS administered on the 16th and 19th day caused no suppression (P control → ALS $\pm$ 2−5 $\leq$ 0.1%).

2. In control mice treated with normal rabbit serum there was no immunosuppression or antigenic competition.

3. A statistically significant difference was demonstrated between the immunity of ALS and NRS-treated groups (P ALS → NRS $\leq$ 0.1%).

Discussion

The above results allowed the following conclusions.

1. Antilymphocyte serum significantly decreases the primary antibody response to tetanus toxoid if immunosuppressive treatment is administered shortly before or after priming.

2. After the establishment of active immunity ALS exerts no immunosuppressive effect.

3. In the mode of action of ALS the antigenicity of heterologous proteins causes no competitive effect.

In the experiments ALS was injected when

(a) the antigen had not yet contacted the immunologically competent cells (5 and 2 days before priming);

(b) the antigen had already contacted the competent cells, but antibody production was yet nil or very slight (2 and 5 days after priming);

(c) there was a manifest antibody production (16 and 19 days after priming).

The definite immunosuppression exerted by ALS given shortly before or after priming may be explained as follows.

It may be supposed that ALS caused lymphocyte depletion [3]. ALS given before priming could prevent the antigen to contact a sufficient number of competent cells.

When ALS was administered after priming the lymphocyte depletion could result in the destruction of primed competent cells. However, for effectively suppressing immunity by lymphocyte depletion or decrease, a chronic lymphopenia should be maintained, for example by thoracic duct drainage. The latter condition is associated with a decrease in resistance and, therefore, had it played an important role in our experiments, the toxic effect of the serum would have predominated. As both the test groups and the animals used for toxicity control remained in normal condition throughout the experiment, it may be supposed that, corresponding to data in the literature, in our animals the decrease in lymphocyte count was only transient.

The immunosuppressive effect of ALS is due, according to many authors, to the adsorption of ALS on the surface of lymphocytes in the form of coating. On the other hand, an excessive blastoid transformation of lymphocytes under the effect of ALS may also be involved [2].

On the basis of our experiments the "blindfolding" theory seems to offer the most probable explanation.

ALS treatment before priming, accordingly, inhibits the recognition of the antigen and/or the uptake of information. Thus the first step of the immunological process is omitted. ALS treatment shortly after the antigenic stimulus might act in a similar manner, although during the period between priming and ALS injection there may be a contact between the competent cells and the antigen. It should be noted that a prolonged resorption of antigen from the depot maintains some antigenic stimuli also after ALS treatment.

Accordingly, two different mechanisms may be involved. On the one hand, the coating formed on primed cells inhibits the secretion of antibodies and, on the other, the inhibitory effect of ALS prevents the contact of immunologically competent cells with the antigen resorbed later from the depot.

ALS caused no complete prevention of immunological events. This finding may be explained by the considerable prolongation of the release of antigen by using adsorbed vaccine. Thus the antigen may enter the circulation when the competent cells have already been released from the suppressive action of ALS or when the titre of ALS has decreased to a level not suppressing the already regenerated competent cells. Our studies indicate

that the half-life of graft protectivity of ALS is 5—6 days, identical with the *in vivo* half-life of ALS heterologous protein [17].

ALS given on the 16th and 19th day of the experiment caused no suppression. This finding may be explained by the fact that anti-tetanus immunity can be characterized mainly with the amount of circulating antibodies at this moment.

In previous experiments [13, 18] we showed that 14 to 21 days after immunization the antitoxin level did not increase logarithmically. Accordingly, at the time of ALS administration the animals possessed an amount of circulating antibodies sufficient to survive the toxin challenge even if ALS reacted with the lymphocytes.

The suppressibility with ALS of secondary antigenic stimulus and immunological memory will be the subject of further experiments.

In the present study it has been shown that ALS is highly effective in suppressing the development of primary antitoxic immunity. The inhibition is not associated with antigenic competition.

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THE EFFECT OF BORDETELLA PERTUSSIS VACCINE ON THE HISTAMINE METABOLISM OF RAT TISSUES

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Summary. Under the effect of *Bordetella pertussis* vaccine (BPV) the histamine level in the tissues and the free histamine content of the urine increased. Parallel with these changes the histamine-destroying activity of the tissues decreased. The peak of these alterations was observed 4 days after vaccination. Histidine decarboxylase activity increased considerably only in the gastric pylorus.

The increase in histamine level has been attributed primarily to the decreased histamine-destroying activity of the tissues. In adrenalectomized animals BPV treatment caused no increase in histamine level or decrease in histamine destruction. Cortisone eliminated or significantly decreased the effect of BPV. It has been concluded that BPV acts indirectly on histamine metabolism by inducing adrenal insufficiency.

It has long been known that *Bordetella pertussis* vaccine (BPV) increases the sensitivity of rats and mice to histamine [1, 2]. The increased sensitivity appears as soon as 24 hours after treatment, its peak is on the 4th or 5th day and, depending on the dose applied, it lasts for 8 to 18 days.

An increased histamine sensitivity has been shown in adrenalectomized rats [3], too, and other alterations are also similar in adrenalectomized and in BPV-treated animals [4, 5], Adrenalectomy induces deep changes in the histamine metabolism of the tissues, increases considerably the histamine level in different rat tissues and decreases their histamine-destroying activity [6, 7]. Histamine toxicity is probably associated with endogenous histamine levels [8] and the actual amine metabolism of the tissues.

In the present work we examined the effect of BPV on the histamine level in rat tissues, on histidine decarboxylase activity, histamine-destroying activity and free histamine content of the urine.

As our results indicated that BPV treatment caused alterations in tissue histamine metabolism similar to those observed after adrenalectomy, we have supposed that BPV treatment causes adrenal insufficiency and thus exerts an indirect action on tissue histamine metabolism. In order to confirm this theory we examined the effect of BPV on adrenalectomized and cortisone-treated rats.

Materials and methods

Wistar rats of both sexes weighing 200 to 250 g were used. The animals were fed on a standard diet and were given water *ad libitum*.

BPV was given in a single intraperitoneal injection containing 9×10^9 organisms. Different groups of animals were sacrificed 1, 4 and 12 days after the injection, then histamine level, histidine decarboxylase activity and histamine-destroying activity in the tissues were determined. Cortisone-treated rats received 10 mg per kg of cortisone (Adreson, Organon) intramuscularly at two-day intervals over a period of 12 days. Adrenalectomy and sham-operation of the control animals were performed in hexobarbital anaesthesia.

BPV was injected 8 days after the operation or treatment to one group of adrenalectomized and to one group of cortisone-treated animals. The rats were sacrificed 12 days later and histamine level and histamine-destroying activity of the tissues were determined. The saline-treated sham-operated control animals were sacrificed 4 days after BPV injection.

Determination of tissue histamine level was performed as described by SHORE *et al.* [9]. For histidine decarboxylase activity the method of TELFORD and WEST [10], for histamine-destroying activity the method of COHN and SHORE [11] was used. Amine levels were expressed as μg histamine basis per g or ml tissue. Each experimental result presented indicates the average of data obtained in at least 10 animals and the corresponding standard deviations. Significance was estimated by STUDENT's *t* test.

Free histamine excretion with urine was determined separately for male and female rats in view of the considerable sexual difference in this respect [12]. One week before the experiment the animals were placed into metabolic cages in order to make them accustomed to the special circumstances. Before beginning the 24 hour urine collection 1 ml 5 *N* HCl was pipetted into the vessel to inhibit bacterial and chemical destruction of histamine. Free histamine content was determined as described by CODE [13]. Average values for 3 days prior to BPV injection were regarded as controls. After injecting BPV, free histamine values were determined in the subsequent 24 hours and on the 4th, 8th and 12th day. The experiments were carried out in 5 female and 5 male rats. Significance was estimated by the *t* test.

Results

Twenty-four hours after BPV injection a significant increase in histamine level was observed only in the gastric pylorus. After 4 days every examined rat tissue showed levels significantly higher than the normal (Fig. 1). With the exception of the lungs, after 12 days the histamine concentration decreased to the original value.

Histidine decarboxylase activity was determined only in the liver, pylorus and duodenum as the activity of this enzyme is very low in other tissues and can be measured only by the isotope technique. The activity increased considerably in the pyloric area as soon as after 24 hours; similarly increased levels were demonstrated on the 4th and 12th days (Fig. 2). Although the decrease in the liver and the increase in the duodenum differed significantly, we did not regard these as important changes.

More definite alterations were revealed in the histamine-destroying activity of the tissues (Fig. 3). The decrease was appreciable after one day and became significant on the 4th day. Values measured on the 12th day did not differ from the control levels.

Female and male rats differed in absolute values for free histamine excretion, but after BPV injection the changes tended to be similar in both sexes (Table I). The free urinary histamine content increased after BPV injection to a peak on the 4th day, then decreased gradually. The changes were definite in both sexes.

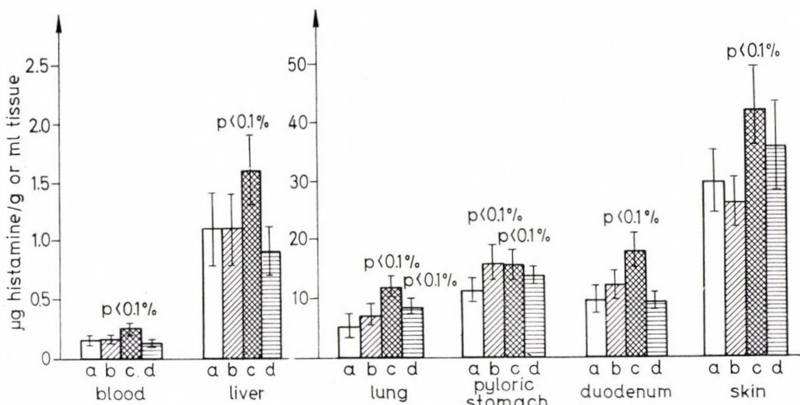

Fig. 1. **Effect of BPV on histamine level in rat tissues. a = control; b = 24 hours after BPV; c = 4 days after BPV; d = 12 days after BPV**

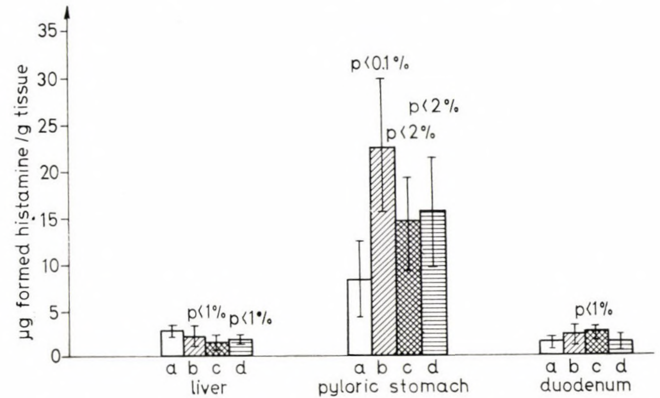

Fig. 2. **Effect of BPV on histidine decarboxylase activity in rat tissues. See legend of Fig. 1**

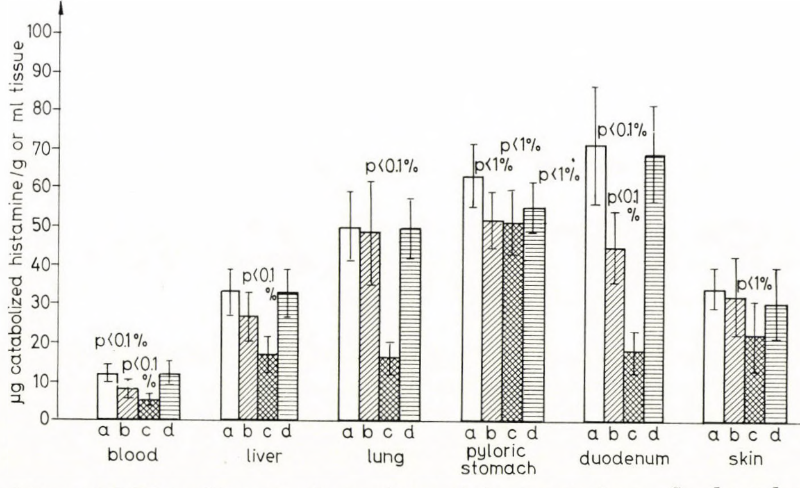

Fig. 3. **Effect of BPV on histamine-destroying activity in rat tissues. See legend of Fig. 1**

Table I

Effect of Bordetella pertussis vaccine on the free histamine content of rat urine

Designation and sex of rats	Free histamine excretion as μg histamine basis per 24 hours				
	Control	1st day	4th day	8th day	12th day
1 ♀	6.5	8.0	9.9	8.7	4.6
2 ♀	6.2	11.3	19.2	12.0	5.2
3 ♀	4.2	4.0	7.4	8.9	3.5
4 ♀	3.7	8.0	8.6	7.9	2.8
5 ♀	5.4	8.7	12.8	8.3	3.0
Average ± S.D.	5.2±1.2	8.0±1.8 P<5%	11.8±4.2 P<2%	9.2±1.5 P<1%	3.8±1.1
6 ♂	0.6	1.3	2.2	1.4	0.7
7 ♂	1.0	1.1	1.6	1.0	0.4
8 ♂	0.7	1.6	1.6	1.1	0.6
9 ♂	0.9	1.7	2.3	1.0	0.6
10 ♂	0.9	1.3	2.1	1.6	0.5
Average ± S.D.	0.8±0.2	1.4±0.2 P<2%	2.0±0.3 P<1%	1.2±0.3 P<5%	0.6±0.1

Fig. 4. Effect of BPV on histamine content of tissues in cortisone-treated rats. a = control; b = BPV; c = cortisone; d = cortisone + BPV. Significance was related to cortisone-treated animals

Cortisone treatment decreased the histamine level in all organs except the pylorus (Fig. 4). This finding was in accordance with previous observations [7]. When cortisone and BPV were given simultaneously, there was no increase in histamine levels but, paradoxically, the histamine content of the duodenum decreased.

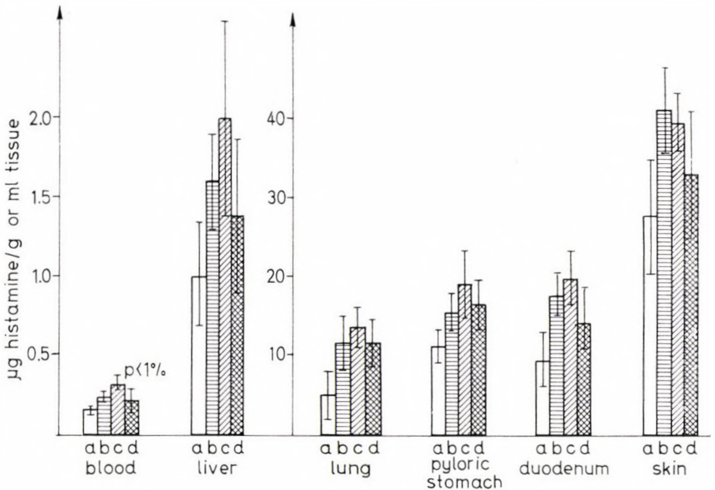

Fig. 5. Effect of BPV on histamine content of tissues of adrenalectomized rats. a = sham operation; b = sham operation + BPV; c = adrenalectomy; d = adrenalectomy + BPV. Significance was related to adrenalectomized animals

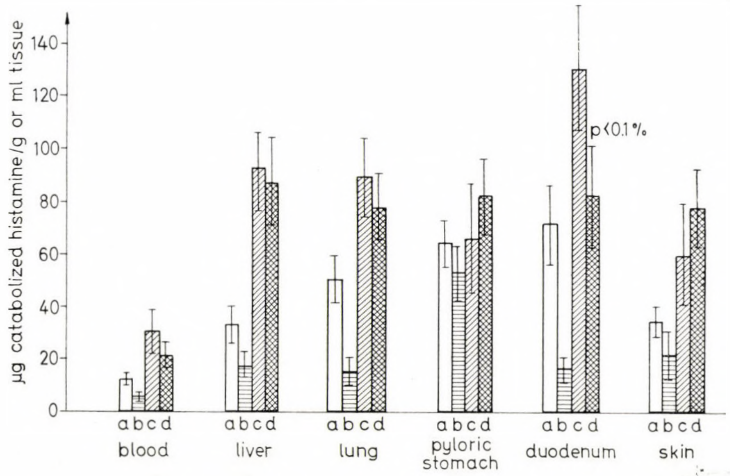

Fig. 6. Effect of BPV on histamine-destroying activity in tissues of cortisone-treated rats. See legend of Fig. 4. Significance was related to adrenalectomized animals

The tissue histamine level was increased by adrenalectomy even more definitely than by BPV. In adrenalectomized animals BPV exerted no histamine-increasing effect; the blood histamine level was significantly lower than in adrenalectomized rats not treated with BPV (Fig. 5).

In cortisone-treated animals the tissues showed a high histamine-destroying activity. BPV injection in cortisone-treated rats decreased histamine

destruction only in the liver and duodenum, but the values were considerably higher than in rats injected with BPV only (Fig. 6).

Adrenalectomy, similarly to BPV, definitely decreased the histamine-destroying activity of the tissues (Fig. 7). BPV, in contrast, exerted no influence on tissue histamine destruction (with the exception of the duodenum).

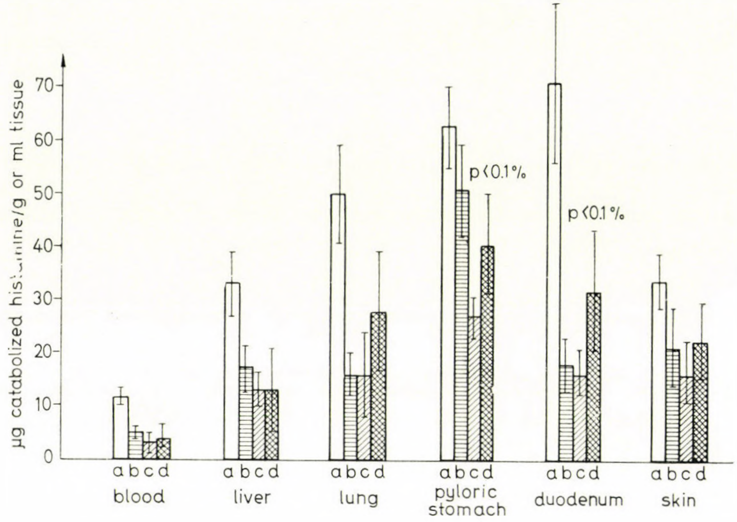

Fig. 7. Effect of BPV on histamine-destroying activity in tissues of adrenalectomized rats. See legend of Fig. 5. Significance was related to adrenalectomized animals

Discussion

The present experiments have shown that BPV injection significantly increases the histamine level in rat tissues. The increase is associated primarily with the decrease of tissue histamine-destroying activity. MATSUI *et al.* [14] have shown that BPV treatment decreases the histamine-destroying activity of the rat lung. The present experiments revealed a similar change in a number of other tissues. The increase in histamine level may be associated with an increased histidine-decarboxylase activity only in the gastric pylorus. As histidine decarboxylase in the pylorus is mainly an inducible enzyme [15], it may be supposed that BPV exerts its effect by enzyme induction, as it has been demonstrated in mice [16]. Changes in histamine anabolism and catabolism are in agreement with our results on free histamine excretion. The increased urinary free histamine level is explained by the decreased destruction and by the increased production of histamine in some tissues, mainly in the pylorus. The difference in histamine excretion between male and female rats reflects probably a sexual difference in histamine catabolism [17].

Adrenalectomy and BPV caused very similar changes in histamine metabolism. In previous studies we have shown that increased tissue histamine levels are associated with a decrease in histamine-destroying activity [7]. The effect of BPV on histamine metabolism can be eliminated or significantly decreased with cortisone. In adrenalectomized animals BPV fails to exert an amine level-increasing and tissue histamine metabolism-decreasing effect. Adrenal insufficiency in BPV-treated rats and mice is indicated also by an increased sensitivity to stressors [5, 18, 19], prolonged hypoglycaemia [20] and decreased vitamin C content of the adrenal cortex [21]. It is also known that adrenalectomized and BPV-treated animals show an increased sensitivity to 5-hydroxytryptamine [22, 23]. Accordingly, it may be concluded that after BPV treatment an adrenocortical insufficiency develops which induces changes in tissue histamine metabolism.

As related to its action in normal animals, BPV causes opposite changes in histamine blood level and histamine-destroying activity in adrenalectomized animals and in the histamine content of the duodenum in cortisone-treated animals. This additional (probably direct) effect of BPV cannot yet be interpreted.

As to the increase in histamine sensitivity of rats and mice under the effect of BPV injection some theories have been advanced. Fishel *et al.* [24] and Stronk and Pittmann [25] attributed importance to the direct effects of the vaccine. The role of a change in tissue histamine metabolism was suggested by Matsui *et al.* [14] and Schayer and Ganley [16]. Based on mouse experiments, the latter authors emphasized the importance of the increased activity of inducible histidine decarboxylase. In our opinion the change in tissue histamine metabolism may play a role in the development of increased histamine sensitivity, but in rats these changes are mainly due to a decrease in histamine-destroying activity. This conception is supported by the fact that the peak changes coincide with the maximum of histamine sensitivity. The increased endogenous histamine level and histamine sensitivity may be parallel [8] and may play a part in the increased histamine sensitivity due to BPV. For a final evidence of the importance of the decrease in histamine decomposing activity further studies are needed on the role of various enzymes involved in histamine catabolism.

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FATTY ACIDS IN STAPHYLOCOCCUS AUREUS AND ESCHERICHIA COLI CULTURED IN VIVO

By

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Summary. (i) The cytological picture of peritoneal exudate in guinea pigs varies with the inducing agent. After the injection of 1% India ink, 94—96% polymorphonuclear neutrophils and 4—6% lymphocytes were demonstrated. Dextran (4%) induced the appearance of 70—80% polymorphonuclear and 14—24% mononuclear leukocytes, and 4—8% lymphocytes. Ten per cent casein induction resulted in a similar distribution of white blood cells with the additional appearance of 8—10% epithelial cells.

(ii) Distribution of total fatty acids in peritoneal exudate was: C_{12} or shorter chain fatty acids, 21—66%; palmitic acid, 10—23%; stearic acid, 9—28%; unsaturated fatty acids, 8—19%.

(iii) Of the total fatty acid content of *Staphylococcus aureus* cultured *in vivo*, 23—42% corresponded to unsaturated fatty acids. The main fatty acids were: anteiso-C_{15} acid, 10—25% palmitic acid, 12—19%; stearic acid, 6—28%; oleic acid, 12—22%; linoleic acid, 6.1—14%; arachic acid, 3—11%.

(iv) In the fatty acid spectrum of *Escherichia coli* cultured *in vivo*, cyclopropane fatty acids were detected only in traces. The main components were: palmitic acid, 24.8%; stearic acid, 22.2%; oleic acid, 15.7%; linoleic acid, 12.4%.

The fact that the virulence of pathogenic bacteria can be enhanced by passage in susceptible animals indicates that metabolites associated with virulence are produced in greater amounts *in vivo* than *in vitro*. As biochemical and biological studies require large quantities of pure bacterial preparations, little is yet known about metabolites and metabolic processes characterizing the increase of virulence *in vivo*. According to GELLENBECK [1] the metabolism of staphylococci increases under conditions *in vivo*. BEINING and KENNEDY [2] showed in 1963 that, as compared to cultivation *in vitro*, *Staph. aureus* produced *in vivo* higher amounts of desoxyribonuclease, alpha haemolysin, leukocidin, and hyaluronidase, changed its sensitivity to gamma globulin, increased its mouse and rabbit pathogenicity and developed two new antigens. SEGAL *et al.* [3] in comparing *M. tuberculosis* cultured *in vitro* and *in vivo* showed that bacteria harvested from mouse lung contained about 25% more total lipids and in their fatty acid spectrum short carbon-chain compounds predominated. VÁCZI *et al.* [4] demonstrated that the lipid and fatty acid composition of staphylococci depended upon the ingredients of the medium, its pH and temperature. In previous papers [5, 6] we have shown that *Staph. aureus* infection caused a disturbance in the host's lipid metabolism.

In the present work we report on studies on the fatty acids of bacteria cultured in the peritoneal cavity of guinea pigs.

Materials and methods

Organisms. Staph. aureus strains 53 and 80/81 were isolated in our institute from human material. *E. coli* O111 strain 30006 was obtained from the Culture Collection, National Institute of Public Health, Budapest.

Cultivation in vivo. Group 1. Guinea pigs weighing 500—600 g were injected intraperitoneally with approximately 2×10^{10} cells of 18 hour cultures of *Staph. aureus* strains 53 and 80/81. The animals died after 12—14 hours, but the peritoneal cavity was opened only after 18 hours. Then the exudate was harvested, the peritoneal cavity was washed twice with 10 ml aliquots of pH 6 saline. After uniting the exudate ultrasonic treatment was performed in a MSE homogenizer (1.5 watt/cm^2), then the material was centrifuged at 4 °C and 3000 r.p.m., washed twice with 4 °C distilled water, resuspended in acetone and refrigerated at 4 °C for 24 hours.

Group 2. Guinea pigs were infected as described for Group 1 and were sacrificed 6 hours after the injection. Harvesting and treatment of bacteria were carried out as in Group 1.

Group 3. The 18 hour culture of *Staph. aureus* strain 80/81 was centrifuged at 4 °C and 3000 r.p.m. for 20 minutes. The deposit was washed, then suspended in saline to the original volume. In order to inhibit phagocytosis, each animal was given intraperitoneally 1 ml per 100 g body weight of 4% dextran 4 hours before infection. Then approximately 2×10^{10} living cells were injected intraperitoneally. The animals were bled 18 hours after the infection. The exudate was collected and the peritoneal cavity was washed twice with isotonic citrate. After uniting the fractions, the bacteria were subjected to differential centrifugation as described by GELLENBECK [1].

Group 4. In vivo cultivation of *E. coli* was performed as indicated for Group 3, with the exception that the animals received 1% dextran which inhibited satisfactorily the phagocytosis.

Fatty acid synthesis by *E. coli in vitro* was examined in 1% dextrose-Casitone (Difco) and in 5% human serum-Casitone (Difco) media.

Each group consisted of 3×6 animals and each determination was performed with bacteria harvested from 6 animals.

Cytological examination of peritoneal exudates. The control animals were divided into 3 groups of 12 each. Each examination was performed with the pooled cells of 4 animals. Group 1 received sterile 1% casein medium (Difco) containing 1% India ink, 1 ml per 100 g weight. Group 2 was given sterile 10% casein medium, Group 3 sterile 4% dextran, 1 ml per 100 g weight, intraperitoneally.

The animals were sacrificed 6 hours after the intraperitoneal injection, the exudate was harvested and the peritoneal cavity was washed twice with 10 ml isotonic citrate. Cell counts were determined in a Bürker chamber and quantitative distribution of the cells was examined after May—Grünwald—Giemsa staining.

The exudate was sedimented at 4 °C, the cells were washed twice in saline, resuspended in acetone and stored at 4 °C for 24 hours. Lipid extraction and fatty acid analysis were performed as described in [7].

Results

When examining the cellular material of bacteria cultured *in vivo*, the preparation should not contain larger amounts of materials originating from the host. Accordingly, at the beginning of the experiments various substances were given intraperitoneally to guinea pigs and the distribution of cells in the resulting exudate and fatty acid composition were determined (Table I).

From Table I it is evident that cell counts were similar (5×10^6 to 9×10^6 per ml) in exudates induced by different substances. The distribution of cells, however, showed remarkable differences. One per cent India ink produced 94—96% polymorphonuclear leukocytes and 6% lymphocytes. Injection of 4% dextran resulted in the appearance of about 75% polymorphonuclear leukocytes; the proportion of lymphocytes was the same as with India ink,

but 14 to 24% mononuclear phagocyting cells appeared. Ten per cent casein induced 73% polymorphonuclear leukocytes, 12% mononuclear leukocytes, 6% lymphocytes and 9% of the cells were characterized by a foamy nuclear structure and slightly basophilic cytoplasm. These cells were probably epithelial in origin.

Table I

Percentage distribution of cells in guinea pig peritoneal exudate

Inducing agent	Cell count	Polymorphonuclear leukocytes	Lymphocytes	Mononuclear leukocytes	Other cells
1% India ink	9×10^6	94—96	4—6	—	—
4% Dextran	5×10^6	70—80	4—8	14—24	—
10% Casein	7×10^6	71—75	5—7	11—13	8—10

Ranges of 3 parallel determinations

Table II

Percentage distribution of fatty acids in peritoneal exudate cells

Fatty acid	Inducing agent		
	1% India ink	4% Dextran	10% Casein
$<C_{12}$	17.0	30.1	59.3
C_{12}	4.8	4.9	7.2
C_{13}	0.8	+	+
$C_{14:1}$	4.4	3.3	5.5
C_{14}	3.5	6.5	5.2
C_{15}	1.7	2.1	3.1
$C_{16:1}$	0.9	+	+
C_{16}	23.7	20.1	9.8
C_{18}	1.8	1.0	1.6
$C_{18:2}$	3.1	1.7	+
$C_{18:1}$	10.2	5.5	2.9
C_{18}	28.3	22.8	9.0

Mean values of 3 parallel determinations

In Table II the fatty acid analysis of the exudates is presented. Exudates induced by different stimuli contained cells different in fatty acid composition. In the peritoneal exudate fatty acids occurred in the following proportions: substances with less than 12 carbon atoms, 17—59%; C_{16}, 9.8—23.7%, C_{18}, 9—28.3%; C_{12}, 4.8—7.2%; C_{14}, 3.5—6.5%; $C_{18:1}$, 3—10.2%; $C_{18:2}$, 0.2—3.1%. Thus the fatty acid spectrum was characterized mainly by the

presence of short carbon chain, C_{16} and C_{18} compounds. Fatty acids characteristic of bacteria (branched chain, cyclopropane, C_{20} or longer carbon chain substances) were not demonstrated.

These data were determined in order to check bacterial preparations cultured *in vivo* in case of a possible contamination with guinea pig fatty acids.

Table III

Percentage distribution of fatty acids in Staph. aureus 80/81 cultured in vivo

Fatty acid	Cultivation for		
	6 hours*	18 hours*	18 hours + 4% dextran**
C_{12}	0.6	0.9	1.3
C_{13}	1.4	—	1.0
$C_{14:1}$	2.2	0.3	2.5
C_{14}	1.9	0.7	1.8
aiC_{15}	25.7	10.1	10.1
C_{15}	—	0.3	1.8
$C_{16:1}$	1.1	0.6	—
C_{16}	12.6	17.3	19.2
C_{17}	0.8	0.3	—
$C_{17:1}$	—	—	1.8
aiC_{17}	2.9	1.4	2.0
$C_{18:2}$	6.1	14.4	6.1
$C_{18:1}$	11.9	22.2	12.6
C_{18}	16.8	25.3	28.3
$C_{19:1}$	1.3	—	—
C_{19}	0.9	—	—
$C_{20:2}$	6.5	4.2	—
C_{20}	7.3	2.6	10.8
Total unsaturated	29.1	42.0	23.0

Mean values of 3 parallel determinations.
* Prepared with ultrasonic treatment.
** Prepared with differential centrifugation.

Table III presents fatty acid distribution in *Staph. aureus* strain 80/81. Bacteria cultured in different manners and for different times contained the same fatty acids. The quantitative distribution, however, was different. In animals infected with whole culture, anteiso-C_{15} fatty acid was demonstrated in 25.7%. Further main components were: stearic acid, 16,8%; palmitic acid, 12.6%; oleic acid, 12%; linoleic acid, 6%. Unsaturated compounds comprised 29% of the total fatty acid content. Bacteria harvested

after 18 hours contained only 10% anteiso-C_{15}. Stearic acid represented 25% of the total fatty acids; oleic and linoleic acids increased considerably (22.2 and 14.4%, respectively). There was no essential change in the proportion of palmitic acid (17.3). The main difference consisted in the increase in unsaturated fatty acids (42% of total fatty acids). Infection with washed bacteria resulted after 18 hours in the following distribution of fatty acids: stearic, 28.3; palmitic, 19.2; unsaturated, 23%; anteiso-C_{15}, 10%.

Staph. aureus 53 cultured *in vivo* showed essentially the same spectrum as strain 80/81. Anteiso-C_{15} occurred in 17.5%; oleic and linoleic acids were present in somewhat higher amounts and unsaturated compounds were detected in 36.8%.

Table IV presents fatty acid distribution in *E. coli* 30006 cultured under different conditions. In bacteria grown in Casitone medium palmitic acid occurred in 40%, cyclopropane compounds in 32%, while unsaturated fatty

Table IV

Percentage distribution of fatty acids in different E. coli cultures

Fatty acid	Casitone (Difco) + 1% glucose	Casitone (Difco) + 5% human serum	*In vivo* + 1% dextran
C_{12}	4.6	3.6	2.1
C_{13}	—	—	2.1
$C_{14:1}$	+	6.6	+
C_{14}	10.8	8.0	2.9
aiC_{15}	2.5	1.6	2.3
C_{15}	0.9	1.7	2.0
beta-OH—C_{14}	6.2	1.9	+
$C_{16:1}$	3.4	1.5	5.3
C_{16}	39.5	43.4	24.8
ΔC_{17}	21.6	11.9	2.5
$C_{17:1}$	—	—	3.9
aiC_{17}	—	—	1.8
$C_{18:2}$	+	3.0	12.4
$C_{18:1}$	+	4.0	15.7
C_{18}	—	4.3	22.2
ΔC_{19}	10.5	5.7	+
Total unsaturated	3.4	15.1	37.3

Mean values of 3 parallel determinations.
ai = anteiso fatty acid.
Δ = cyclopropane fatty acid.
Figure after colon = number of unsaturated bonds.

acids only in 3.4%. In 5% human serum-Casitone medium cultures unsaturated fatty acids were present in 15.1% and the amount of beta-hydroxymyristic acid and cyclopropane compounds was considerably less than in simple Casitone medium cultures.

If cultures made *in vitro* and *in vivo* are compared, it is striking that in the latter high amounts of unsaturated fatty acids occurred while cyclopropane fatty acids were practically absent. Fatty acids containing less than 16 carbon atoms comprised 23 to 25% of the total fatty acids *in vitro* but only 11% *in vivo*. The amount of palmitic acid was nearly 40% less in the latter. These results indicate that *Staph. aureus* and *E. coli*, if grown in protein (lipoprotein)-containing medium, increase their unsaturated fatty acid content. During cultivation *in vivo* the increase in unsaturated fatty acids is still higher.

Discussion

Our results have confirmed that the lipid composition of bacteria depends on cultural conditions [7—11]. In the interpretation of the present data it should first of all be considered whether the observed changes really reflect lipid metabolism alterations in the test bacteria or are due to contamination originating from the host.

Fatty acid determination in cells in peritoneal exudates induced by different agents showed that mainly fatty acids containing less than 12 carbon atoms and C_{12} compounds were present in high amounts (21—66%). Unsaturated fatty acids were detected in 8—19%. In guinea pig cells fatty acids characteristic of bacteria (anteiso, cyclopropane, C_{20} or longer chain compounds) were absent. These data allowed to conclude that our methods were suitable for producing pure bacterial preparations and, therefore, it is justified to assess changes in the lipid metabolism of bacteria. In bacteria cultured *in vivo* the proportion of unsaturated fatty acids was 23—42%. Thus, bacteria seem to be able to incorporate shorter carbon-chain fatty acids from the environment.

The observed changes in lipid metabolism have confirmed that bacterial metabolism increases *in vivo* [1, 2]. Accumulation of unsaturated fatty acids causes the loosening of the membrane structure, which may be favourable for the increase in metabolic processes.

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EFFECT OF EMETINE ON THE MULTIPLICATION OF PSEUDORABIES AND SEMLIKI FOREST VIRUSES AND ON THE INTERFERON PRODUCTION BY CULTURED CELLS

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Summary. Emetine and 2-dehydroemetine were found to inhibit multiplication of pseudorabies and Semliki Forest viruses in concentrations inhibiting cell division in chick embryo fibroblast cultures. The initial period of viral multiplication cycle is the most sensitive to the effect of the drugs. Interferon synthesis induced by adenovirus type 8 in chick embryo fibroblasts is inhibited by one-tenth of the concentration inhibiting viral growth. The effect of interferon is inhibited by emetine at low concentrations. Neither emetine nor 2-dehydroemetine influenced the survival of pseudorabies-infected mice.

The antiviral effect of emetine and 2-dehydroemetine might be explained by the protein-synthesis-blocking effect of these drugs. It is supposed that the favourable clinical results obtained with emetine in some human viral diseases cannot be explained by the antiviral action of the drug.

Emetine is one of the alkaloids of *Uragoga ipecacuanha*. Its expectorant and emetic effect has been known for long. Due to their amoebocidal activity, both emetine and its less toxic derivative, dehydroemetine, are widely applied in the therapy of *Entamoeba histolytica* infections. Moreover, there are data suggestive of an antibacterial action of emetine [1—4] and an antimycotic action of dehydroemetine [5, 6]. Favourable results have been obtained with emetine in the treatment of nonspecific granulomas [8] and in the local treatment of scorpion bite [12]. Emetine has been found antiphlogistic [10, 11]. Dehydroemetine was effective in the therapy of schistosomiasis [7]; it gave rise to remission in cases of chronic myeloid leukaemia [9]. Emetine was found mutagenic for Corynebacterium [13].

A therapeutic effect by emetine in viral disease was first observed by VIDAL [14]. His results have been confirmed and completed by further data [10, 15—18]. Most authors attribute the favourable effect primarily to the antiphlogistic action of the drug, but a virocidal or virostatic effect has also been proposed [10, 11].

Data being scarce on the virus-inhibiting effect of emetine and dehydroemetine [19—21] and as to our best knowledge chemotherapeutic studies *in vitro* have not been carried out, we have investigated the effect of emetine and dehydroemetine on the multiplication of pseudorabies and Semliki Forest viruses.

Materials and methods

The chick embryo fibroblast (CEF) monolayers and tube cultures, and the composition of the media used have been described elsewhere [22].

Viruses. The pseudorabies virus and type 8 adenovirus were kindly supplied by Dr. I. Béládi (Szeged), the Semliki Forest virus by Dr. I. S. Portefield (London). Pseudorabies virus and Semliki Forest virus were propagated in CEF cultures, adenovirus was propagated in HEp-2 cells.

Materials. A commercial emetine preparation and 2-dehydroemetine (rac-2-dehydro-emetine-chlorohydrate-hydrate, kindly supplied by the Research Institute for Pharmaceutical Chemistry, Budapest) were used.

The drugs were dissolved in distilled water and diluted to a concentration of 1 mg/ml. Further dilutions were made from these stock solutions.

Toxicity tests. The cytotoxic effect of varying drug concentrations was examined in CEF monolayers and tube cultures, and the inhibition of interferon production was tested in CEF cells [23].

Interferon production and assay. To produce interferon, adenovirus type 8 was added to one-day-old CEF monolayers [24, 25]. Interferon production was estimated in CEF monolayers by the plaque-reduction test, using Semliki Forest virus as challenge virus.

Inhibition of interferon production. Adenovirus type 8 was allowed to adsorb to CEF monolayers for 2 hours at 37 °C. The monolayers were then washed three times with Hanks' balanced salt solution, and 3 ml medium containing varying amounts of alkaloid was added. Interferon production was determined after incubation at 37 °C for 48 hours.

Inhibition with emetine of the action of interferon. Hanks' solution containing 5 U/ml interferon and varying concentrations of the drug was added to CEF monolayers, 3 ml to each. After four hours incubation at 37 °C the monolayers were washed three times with Hanks' solution and each of them was infected with 150 PFU of Semliki Forest virus, added in a volume of 0.5 ml. After an adsorption period of 2 hours the fluid phase was removed and the cultures were covered with a semisolid overlay. The plaques were counted after 48 hours incubation at 37 °C. For comparison, monolayers were treated with interferon only.

Virocidal effect. One mg of emetine or of 2-dehydroemetine was added to 1 ml of a viral suspension containing 10^8 PFU of virus. The suspensions were then kept for 3 hours in a water bath at 37 °C and titrated by the plaque technique.

Estimation of the antiviral effect by the agar-diffusion test [26, 27]. The method has been described elsewhere [22].

Determination of the antiviral effect of varying concentrations of emetine and 2-dehydroemetine. CEF monolayers were infected at a multiplicity rate of 10 PFU/cell. After an adsorption period of 2 hours at 4 °C the monolayers were washed with Hanks' solution three times. Then 3 ml nutrient medium containing varying amounts of alkaloid was added to each culture. The monolayers infected with Semliki Forest virus and those infected with pseudorabies virus were incubated at 37 °C for 7 and 12 hours, respectively, and subsequently frozen at −25 °C. The cultures were then thawed, pooled, and centrifuged at 4000 *g* for 5 minutes. Virus was determined in the supernatant by the plaque technique.

Inhibition of the one-step growth cycle. CEF monolayers were infected with 10 PFU/cell of virus and the virus was allowed to adsorb for 2 hours at 4 °C. Subsequently, the monolayers were washed with 3 × 3 ml of Hanks' solution. To part of the cultures 3 ml nutrient medium containing 0.3 μg/ml alkaloid, to the remainder alkaloid-free medium of the same volume, was added. At intervals of one hour two cultures from each group were frozen. These were then thawed, the parallels were mixed and centrifuged. The virus in the fluid phase was determined by the plaque technique.

To determine in which period of the growth cycle the alkaloid is active, CEF monolayers were incubated at 37 °C with 2.5 ml nutrient fluid each, and in every hour of incubation two monolayers were supplemented by 0.5 ml nutrient medium containing 1.8 μg/ml alkaloid. The cultures infected by Semliki Forest virus and pseudorabies virus were frozen after 7 and 12-hour incubation, respectively. Virus was determined in the fluid phase by the plaque technique.

Effect of emetine pretreatment on virus multiplication. Monolayers were incubated for various times at 37 °C, each with 3 ml medium containing 0.5 μg/ml alkaloid. Subsequently, each monolayer was washed with 3 ml Hanks' solution three times and infected with 10 PFU/cell virus. After an adsorption period of 2 hours the cultures were washed again and 3 ml medium was added to each one. After incubation for 7 hours (Semliki Forest virus) or 12 hours (pseudorabies virus), the cultures were frozen and virus was determined as described above.

Tests in vivo. Albino mice weighing 20 g on the average, were infected with pseudorabies virus intravenously. The individual dose of virus was 20 LD_{50}. Emetine or dehydroemetine was injected in 0.5 ml saline three times, intraperitoneally: the first dose was given simultaneously with the infection, the second and third doses on the next two days. Control mice were given 0.5 ml of saline and the survival times were compared.

Results

Both emetine and dehydroemetine failed to display a virocidal action.

Emetine and dehydroemetine proved to be approximately equally toxic: 0.2 to 0.3 μg/ml alkaloid slowed down cell division to an appreciable degree. However, 1 μg/ml was required to shorten the survival time of the cultures. Interferon production induced by adenovirus type 8 was inhibited at concentrations as low as 0.02 to 0.05 μg/ml (Table I). This implies that protein synthesis in CEF cells is disturbed even at such low concentrations.

Table I

Effect of emetine and 2-dehydroemetine at different concentrations on interferon production by CEF cells

Alkaloid		Interferon titre
designation	μg/ml concentration	
Nil	—	128
Emetine	0.01	128
	0.02	64
	0.05	32
	0.1	16—32
	0.3	8
	0.5	2—4
	1.0	<2
2-dehydroemetine	0.01	128
	0.02	64—128
	0.05	64
	0.1	32
	0.3	4—8
	0.5	2
	1.0	<2

Like other inhibitors of protein synthesis [28—31], emetine definitely reduced the antiviral effect of interferon; 0.1 μg/ml was found to be the lowest effective dose. Larger doses, besides inhibiting the antiviral effect of interferon, inhibited plaque formation by Semliki Forest virus. The resultants of the two factors are shown in Table II.

Table II

Suppression by emetine of the antiviral effect of interferon in CEF cells

Emetine concentration μg/ml	Plaque count* in the		Suppression of interferon action, per cent**
	absence	presence	
	of interferon		
—	100***	—	—
—	—	0****	—
0.01	100	0	0
0.05	100	<1	<1
0.1	100	8	8
0.2	47	11	23
0.3	9	6	66
0.5	0	0	—

* Average from 4 parallels in per cent related to the control.
** 100 × plaque count in the presence of interferon per plaque count in the absence of interferon.
*** virus control.
**** interferon control.

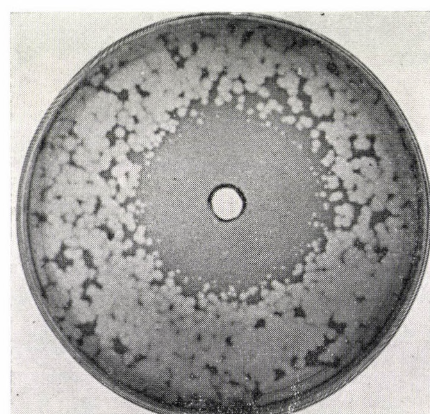

Fig. 1. Effect of 70 μg/ml of emetine on plaque formation by pseudorabies virus

Using the agar-diffusion technique, 150 μg/ml was found to be the lowest toxic concentration of each alkaloid. Fig. 1 shows the effect of 70 μg/ml emetine on plaque formation by pseudorabies virus. A similar degree of inhibition was demonstrable with Semliki Forest virus. The degree of inhibition was the same whether emetine or 2-dehydroemetine was used in the agar-diffusion test.

Using different alkaloid concentrations in the same test, we found that 0.2 μg/ml emetine or 2-dehydroemetine, in a volume of 3 ml, was the lowest concentration to inhibit the multiplication of either of the two viruses (Figs 2 and 3).

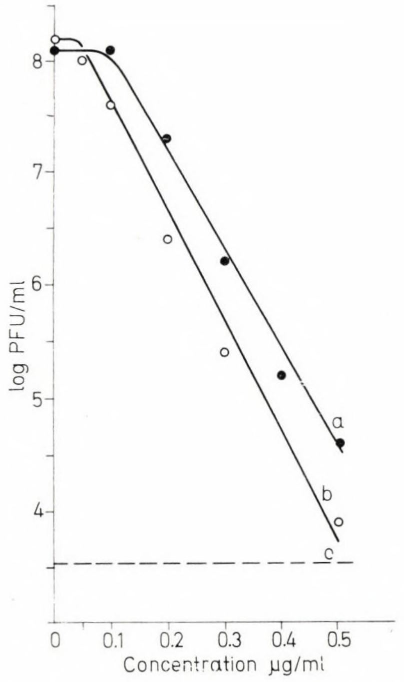

Fig. 2. Effect of emetine and 2-dehydroemetine at different concentrations on the multiplication of pseudorabies virus. a: emetine; b: 2-dehydroemetine; c: adsorbed virus

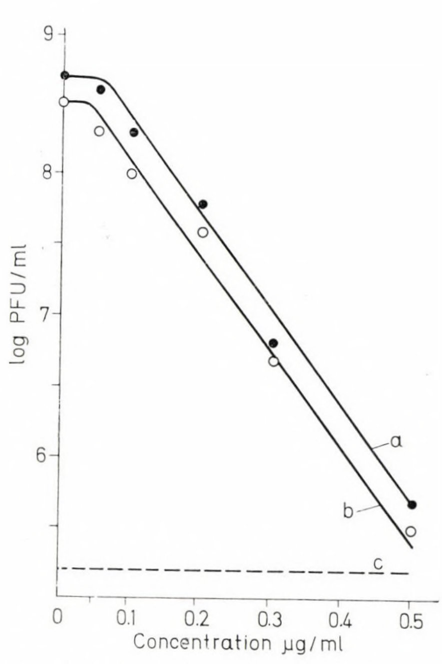

Fig. 3. Effect of emetine and 2-dehydroemetine at different concentrations on the multiplication of Semliki Forest virus. a: emetine; b: 2-dehydroemetine; c: adsorbed virus

As regards the effect on the one-step growth cycle, we have shown that the initial period of the cycle is the most sensitive to the drug. Emetine administered at the start of the cycle significantly prolonged the eclipse period, but did not prevent the replication itself which was, however, less effective than in the control cultures (Figs 4 and 5). If emetine was added 2 hours after the pseudorabies virus or 1 hour after the Semliki Forest virus, inhibition was less pronounced.

When cell cultures were pre-incubated in an emetine-containing medium for one hour, inhibition was more intensive than after a 30-minute pre-incubation. However, a further prolongation of the pre-incubation period failed to increase the inhibition (Fig. 6). This is in good accordance with earlier ob-

servations [21] suggesting that emetine penetrates into the cells and exerts its effect rapidly.

A daily dose of 5 mg/kg of emetine or 10 mg/kg of dehydroemetine on three successive days did not change the life span of noninfected mice. A daily

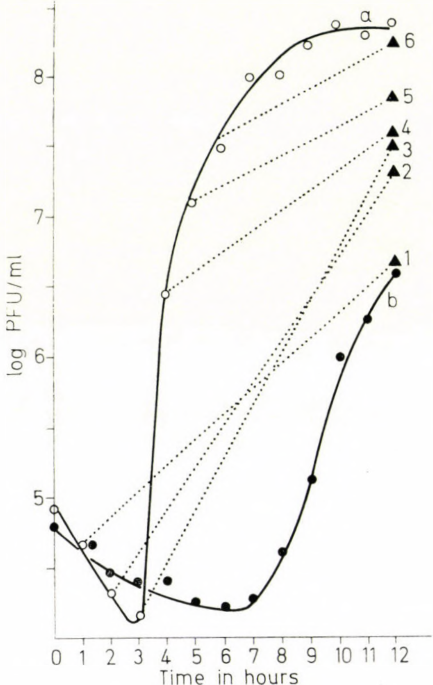

Fig. 4. Effect of 0.3 μg/ml of emetine on the one-step cycle of pseudorabies virus. a: control; b: emetine-treated; 1—6: log PFU/ml in emetine-treated cultures 12 hours after inoculation. The starting point of each dotted line shows the time of emetine addition

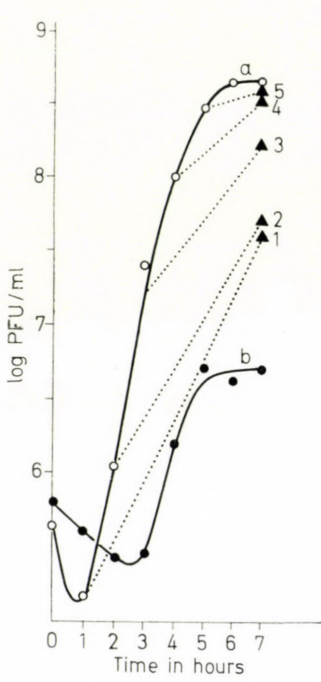

Fig. 5. Effect of 0.3 μg/ml of emetine on the one-step cycle of Semliki Forest virus. a: control; b: emetine-treated; 1—5: log PFU/ml in emetine-treated cultures 7 hours after inoculation. The starting point of each dotted line shows the time of emetine addition

dose of 2.5 mg/kg emetine did not change, whereas 5.0 mg/kg shortened the survival of mice infected with pseudorabies virus. The virus could be reisolated from the brain of the dead mice. The mice given 10 mg/kg dehydroemetine lived longer than the control mice, but the difference could not be appreciated (Table III).

Table III

Effect of emetine and 2-dehydroemetine on the survival of mice infected with pseudorabies virus

Alkaloid		Cumulative death rate, per cent**					
designation	single dose mg/kg*	2	3	4	5	6	7
		days after inoculation					
Nil	—	0	0	40	80	100	
Emetine	0.5	0	0	30	100		
	1.0	0	0	50	70	100	
	2.5	0	10	60	100		
	5.0	0	80	100			
2-dehydroemetine	2.5	0	0	10	80	100	
	5.0	0	0	0	60	100	
	10.0	0	0	0	20	50	100

* Three times intraperitoneally
** Average of two experiments. Each group consisted of 20—30 mice.

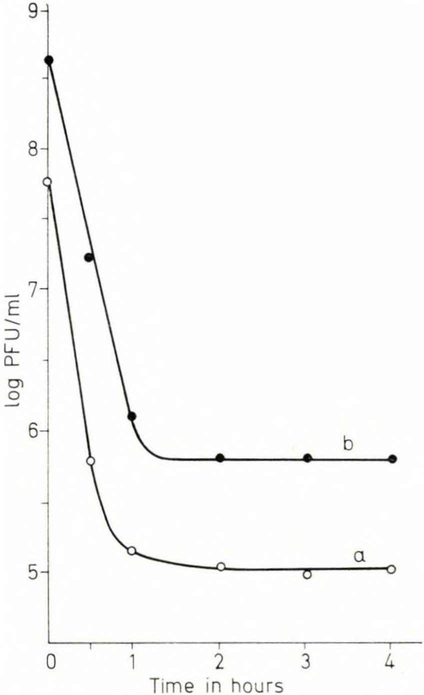

Fig. 6. Effect of emetine pre-treatment on the multiplication of pseudorabies and Semliki Forest viruses. a: pseudorabies virus; b: Semliki Forest virus

Discussion

GROLLMAN [21, 32, 33] demonstrated that emetine selectively inhibits protein synthesis, its point of attack being the same as that of cycloheximide. YOUNGNER *et al.* [34, 35] have shown that cycloheximide inhibits interferon production. We therefore supposed that the inhibitory effect of emetine on interferon production is due to the inhibition of protein synthesis.

We have shown in the present study that interferon production by CEF cells is inhibited by 10 times lower concentrations of emetine or dehydroemetine than the concentrations inhibitory for pseudorabies or Semliki Forest virus. This means that the inhibition of virus multiplication requires alkaloid concentrations seriously injuring protein synthesis.

Like cycloheximide [36], emetine failed to inhibit virus multiplication, except in doses reducing the cell division rate. However, to shorten the life span of the cells, 5 times greater concentrations were required. This may be considered a slightly selective effect. The selectivity of the effect of emetine is supported by GROLLMAN's [21] experiment in which cellular RNA synthesis proved to be much more resistant to emetine than the synthesis of viral RNA.

The alkaloids at a concentration of 0.3 μg/ml reduced interferon production by 94%. This is suggestive of an intensive suppression of protein synthesis. In spite of this virus multiplication proceeded even at such high alkaloid concentrations (Figs 4 and 5). We therefore suppose that in the agar-diffusion test the true toxicity of the alkaloid failed to manifest itself.

GRUNBERG and PRINCE [20] claimed that emetine and dehydroemetine were antiviral *in vivo*. Our experiments with pseudorabies virus failed to confirm this.

The high toxicity and the lack of an effect *in vivo* suggest that the favourable therapeutical results achieved with emetine cannot be attributed to an antiviral effect.

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PROPERTIES OF L-SERINE DEAMINASE FROM SALMONELLA TYPHI-MURIUM AND BACILLUS CEREUS

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Summary. Under the effect of dilution L-serine deaminase of *S. typhi-murium* and *B. cereus* is rapidly inactivated. The rate of inactivation of the *S. typhi-murium* enzyme can be decreased by L-serine. Inactivation of the L-serine deaminase from *B. cereus* depends only on the dilution of the enzyme.

The enzymes are specific for L-serine in both organisms and do not attack other amino acids. Their pH optimum is 8.5; cofactor requirement has not been demonstrated. The dependence of reaction rate on substrate concentration does not follow the simple Michaelis—Menten-type kinetics. Their activity is competitively inhibited by L-cysteine and D-serine.

Our knowledge about bacterial L-serine deaminase is very limited. It was believed for long that L-threonine deaminase and L-serine deaminase were identical enzymes [1]. Boyd and Lichstein [2] and Pardee and Prestidge [3] have shown that L-serine and L-threonine deamination by *Escherichia coli* may be altered independently, in other words the existence of a distinct L-serine deaminase may be supposed. L-serine-specific enzymes were extracted from *Clostridium acidi-urici* by Benziman *et al.* [4] and from *Streptomyces rimosus* by Szentirmai and Horváth [5]. In a previous paper [6] we have shown that L-serine deaminase of *E. coli* is highly specific for L-serine, in substrate saturation experiment it does not follow the simple Michaelis—Menten kinetics, and it is a structurally labile molecule which can competitively be inhibited by D-serine and L-cysteine.

It seemed interesting to investigate therefore whether the high lability of the molecule and anomaly in substrate saturation are characteristic only for *E. coli* enzyme or also for enzymes from other bacteria. In the present paper we describe studies on L-serine deaminase from *Salmonella typhi-murium* and *Bacillus cereus* as compared to the same enzyme of *E. coli.*

Materials and methods

Bacteria. S. typhi-murium isolated in our institute and *B. cereus* strain WS from the collection of Dr. G. Ivánovics were used.

Cultivation. Tryptone-yeast extract medium [6] repressing L-threonine deaminase production and inducing optimal L-serine deaminase production was used. Culturing was performed as described in [6].

Enzyme preparation. Late logarithmic phase cultures were centrifuged, washed with 0.02 *M* phosphate buffer pH 7.5, and resuspended to contain more than 10 mg per ml protein. Then the bacteria were subjected to ultrasonic treatment in ice bath by 60 watt MSE apparatus in 2.5 ml aliquots for 60 seconds. The treated bacteria were used directly for enzyme determination.

Assay of enzyme activity was performed on the basis of FRIEDEMANN and HAUGEN's method [6] by determining pyruvate released at 30 °C. The reaction was terminated after 5 minutes in substrate saturation experiments and after 10 minutes in other experiments. Protein content was determined as described by LOWRY *et al.* [7].

Results

Ultrasonic treatment of *S. typhi-murium* partly inactivates L-serine deaminase. This inactivation is inhibited by L- or D-serine. The effect of ultrasonic treatment on *B. cereus* is independent from the presence or absence of serine (Table I). When sonically-treated cultures were incubated at 30 °C, the enzyme activity of *B. cereus* showed practically no alteration. The activity of *S. typhi-murium* enzyme remained similarly unchanged if treatment had been performed in buffer or in the presence of D-serine. If the bacteria were, however, treated in the presence of L-serine, the activity of the enzyme decreased (Table I).

Table I

L-serine deaminase activity of S. typhi-murium and of B. cereus

Time after ultrasonic treatment, minutes	S. typhi-murium			B. cereus		
	Buffer	Buffer-80 μmoles L-serine/ml	Buffer-300 μmoles D-serine/ml	Buffer	Buffer-80 μmoles L-serine/ml	Buffer-300 μmoles D-serine/ml
0	56.0	94.0	73.5	114.0	114.0	111.0
30	50.0	52.8	66.5	101.0	104.0	97.2
60	45.0	51.4	66.3	90.5	104.0	90.5

Sonically-treated bacterial suspensions were incubated in water bath at 30 °C. Activity determinations were carried out at the indicated intervals. Activity is expressed as amount of pyruvate in mμmoles/ml/10 μg protein produced during 10 minutes.

When the sonicated extracts were diluted the enzymes were rapidly inactivated. The rate of inactivation depended upon the degree of dilution. *S. typhi-murium* enzyme was more sensitive to dilution (Fig. 1A) than *B. cereus* enzyme (Fig. 1B). The kinetics of enzyme inactivation was not influenced if the bacterial extract was diluted with enzyme-free sonically-treated bacterial preparation, or with a solution corresponding in protein concentration to the initial value of the extract. Thus, the inactivation was due to the dilution of the enzyme. The enzyme of *S. typhi-murium* can partially be protected from inactivation by L-serine (Fig. 2A), but the inactivation of *B. cereus* enzyme was not influenced by the presence of the substrate (Fig. 2B). From

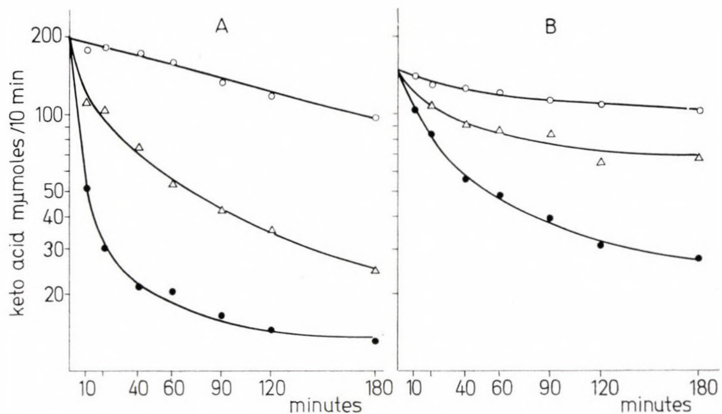

Fig. 1. Inactivation of enzymes by dilution. Sonically-treated bacteria were diluted and incubated in water bath at 30 °C. A: *S. typhi-murium* enzyme. Protein content of suspension during incubation: 16,500 μg/ml (open circles); 1650 μg/ml (open triangles); 410 μg/ml (solid circles). B: *B. cereus* enzyme. Protein content of suspension during incubation: 6000 μg/ml (open circles); 6000 μg/ml (open triangles); 150 μg/ml (solid circles). The curves represent activity decreases in the preparations expressed as 41 μg (A) or 15 μg (B) protein activity

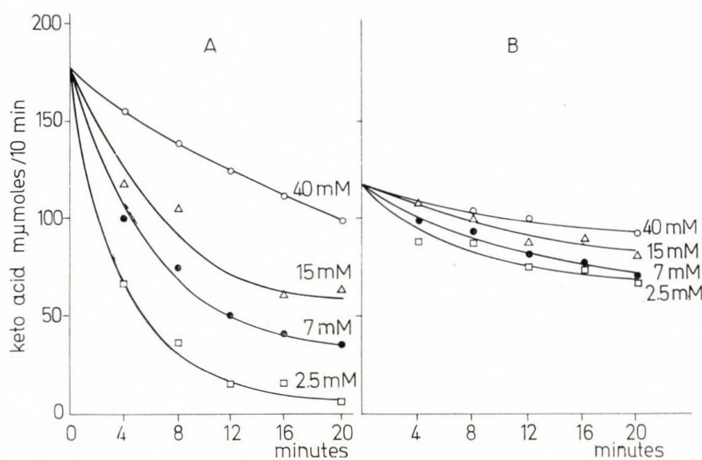

Fig. 2. Inactivation of enzymes by dilution at different L-serine concentrations. A: *S. typhimurium* enzyme; protein, 260 μg/ml. B: *B. cereus* enzyme; protein, 130 μg/ml. The curves represent inactivation of enzymes diluted to identical concentrations but incubated in the presence of different L-serine concentrations at 30 °C. The figures next to the curves indicate L-serine concentrations at the beginning of incubation

Figs 2A and 2B it is evident that, when diluted, both enzymes were partly inactivated even at high substrate concentrations (40 μmoles/ml). However, as shown in Figs 3A and 3B, at high L-serine concentrations the activity of both enzymes was linear. Both enzymes exerted optimum activity at pH 8.5.

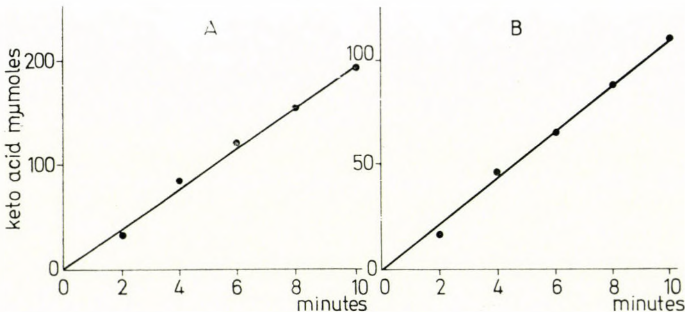

Fig. 3. Keto acid production as a function of time. A: *S. typhi-murium* enzyme; protein, 32 μg/ml. B: *B. cereus* enzyme; protein, 13 μg/ml. Substrate concentration, 40 μmoles L-serine per ml (in both experiments)

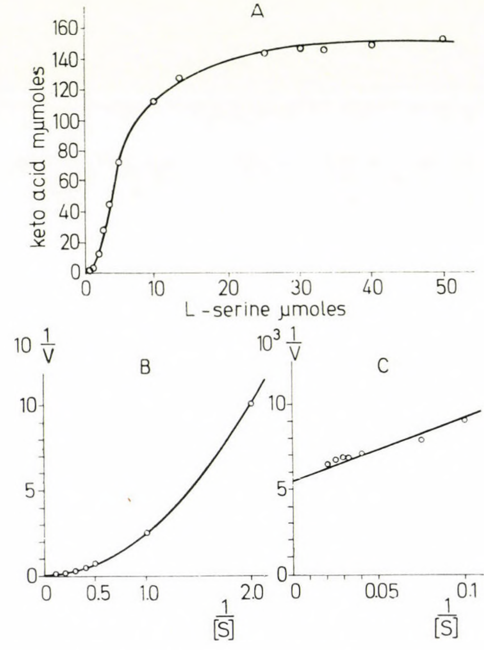

Fig. 4. Substrate saturation experiments, with *B. cereus* enzyme. Reaction time, 5 minutes; protein, 40 μg/ml. A: Keto acid production plotted against L-serine concentration. B: Values obtained in the range of 0.5—13.3 μmoles of L-serine/ml expressed by the double reciprocal method. C: Values obtained int he range of 10—50 μmoles of L-serine/ml expressed by the double reciprocal method

In substrate saturation experiments both enzymes showed sigmoid curves and, if the values were plotted according to the double reciprocal method, no straight lines but concave curves were obtained. If values measured near to the substrate saturation were only plotted, from the resulting straight lines the K_m value could be estimated. These approximated 6.60×10^{-3} *M* for both enzymes with L-serine (Figs 4 and 5).

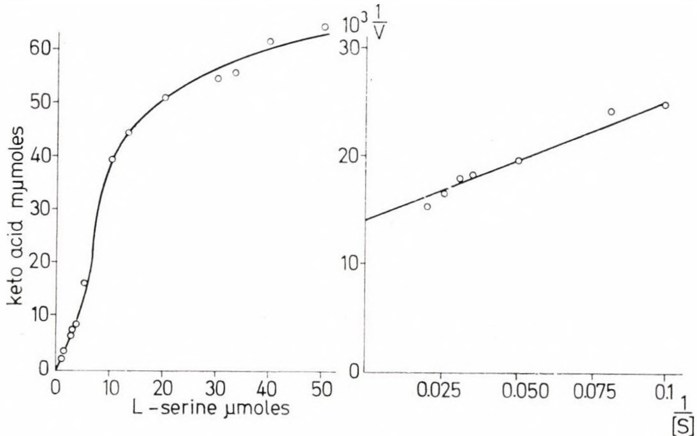

Fig. 5. Substrate saturation experiment, with *S. typhi-murium* enzyme. Reaction time, 5 minutes; protein, 24 μg/ml. Keto acid production plotted against L-serine concentration (left). Values obtained in the range of 10—50 μmoles of L-serine/ml expressed by the double reciprocal method (right)

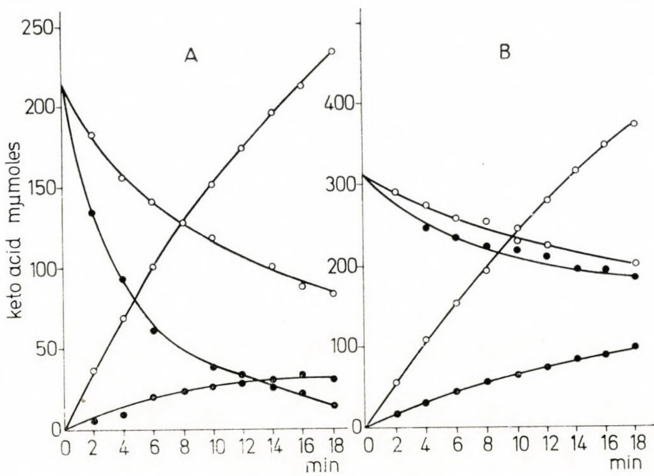

Fig. 6. Simultaneous determination of L-serine deaminase inactivation and substrate breakdown. A: *S. typhi-murium* enzyme, protein, 46 μg/ml. B: *B. cereus* enzyme, protein 30 μg/ml. Effect of diluted enzymes on 7 μmoles (solid circles) and 15 μmoles (open circles) of L-serine per ml at 30 °C. At the indicated intervals, substrate breakdown was terminated with dinitrophenylhydrazine (1.0 ml to 0.1 ml system, curves starting from zero point). At the same time 1.0 ml aliquots were added to 0.1 ml solution containing 40 μmoles L-serine and incubated for further 10 minutes at 30 °C in order to determine the remained activity at saturated substrate concentration (upper curves)

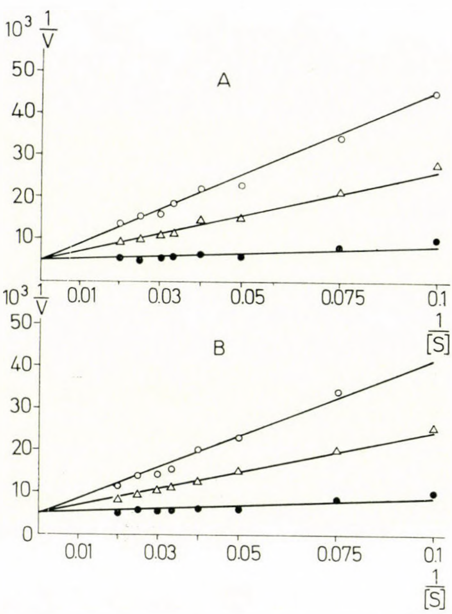

Fig. 7. Inhibition of *S. typhi-murium* enzyme by L-cysteine and D-serine. Substrate concentration, 10—50 μmoles/ml. A: Effect of L-cysteine. Solid circles: control; open triangles: 5 μmoles of L-cysteine/ml. Open circles: 10 μmoles of L-serine/ml. B: Effect of D-serine. Solid circles: control; open triangles: 20 μmoles of D-serine/ml; open circles: 40 μmoles of D-serine/ml

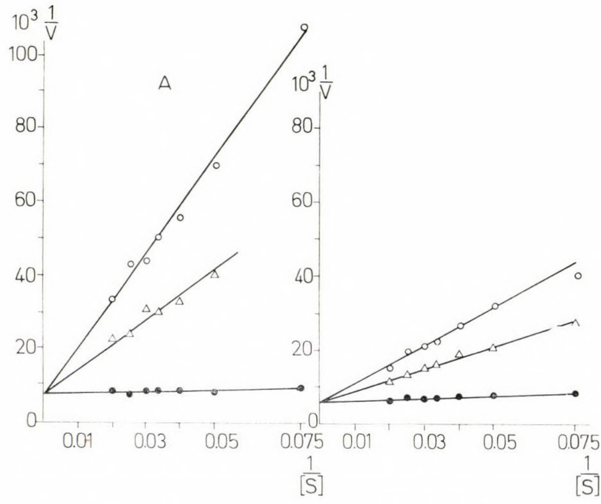

Fig. 8. Inhibition of *B. cereus* enzyme by L-cysteine and D-serine. Substrate concentration, 13.4—50 μmoles/ml. A: Effect of L-cysteine. Solid circles: control; open triangles: 5 μmoles of L-cysteine/ml; open circles: 10 μmoles of L-cysteine/ml. B: Effect of D-serine. Solid circles: control; open triangles: 5 μmoles of D-serine/ml; open circles: 40 μmoles of D-serine/ml

The best explanation of the anomaly of the saturation curves would be that *S. typhi-murium* enzyme is inactivated more rapidly at low substrate concentrations than at high substrate concentrations and, accordingly, at low substrate concentrations the values are smaller than expected. Although the diluted *B. cereus* enzyme is inactivated at a uniform rate in the presence of low and high substrate concentrations (Fig. 2B), the effect of inactivation would be more evident at low than at high substrate concentration. Should this explanation be correct, no linear response could be obtained at low substrate concentrations in the first 5 minutes.

In Fig. 6 it is clearly seen that during the first 6 to 8 minutes enzymatic activity was approximately linear even at low substrate concentrations despite of the simultaneous inactivation of the enzymes. As in substrate saturation experiments 5 minute reaction periods were examined, the sigmoid saturation curves cannot be explained alone with the inactivation of the enzymes.

The result of experiments demonstrated in Fig. 6 is not only insufficient to explain the origin of sigmoid saturation curves but raises another problem, namely, how can such a high degree of inactivation remain latent during initial reaction of the enzyme and substrate? This question, which cannot be answered at present, will be discussed later on. It seems certain, however, that technical errors could be excluded.

At the protein concentrations used (10—100 μg/ml) the enzymes were strictly specific: from the 20 amino acids tested solely L-serine was deaminated. Cofactor requirement was not demonstrated. L-cysteine and D-serine inhibited the activity of these enzymes in a competitive manner (Figs 7 and 8).

Discussion

It has been shown that L-serine deaminase of *E. coli* is a labile molecule which is rapidly inactivated on dilution and does not follow in its activity the normal Michaelis–Menten kinetics [6]. *S. typhi-murium* and *B. cereus* L-serine deaminase behaved in an essentially similar manner. The common properties of the three deaminases can be summarized as follows.

(a) Dilution inactivates all three enzymes. The rate of inactivation of *E. coli* and *S. typhi-murium* enzymes can be decreased with L-serine. In *B. cereus* the rate of inactivation depends only on the degree of dilution and is not appreciably influenced by L-serine.

(b) All three enzymes are specific for L-serine, and their pH optimum is at 8.5. Cofactor requirement has not been demonstrated. The activity of the enzymes is competitively inhibited by L-cysteine and D-serine.

(c) In substrate saturation experiment the enzymes do not follow the simple Michaelis–Menten kinetics.

The dilution lability of the enzymes may be explained by a simple hypothesis: L-serine deaminase is either a polymer molecule irreversibly dissociating upon dilution, or else a labile protein molecule protected by some cellular component until the protein and protective agent are separated as an effect of dilution. These two explanations have been pointed out in a previous paper [6]; either of them may be valid according to the experimental data.

High specificity is characteristic of bacterial L-serine deaminases [4—6]. Although a definite L-serine deaminating activity may be shown by other bacterial enzymes such as purified tryptophanase [8], B protein of tryptophan synthetase [9], and threonine deaminase [10], these enzymes can easily be distinguished from L-serine deaminase on the basis of specificity and cofactor requirement.

Interpretation of the kinetics of enzyme—substrate reaction under the present experimental conditions is a difficult question. It seems probable that the initial reaction is not significantly influenced by the simultaneous partial inactivation of the enzyme. This unexpected remarkable finding is probably due to an uncommon substrate — enzyme relation. In order to perform adequate kinetic experiments, highly purified enzymes are needed. All our attempts, however, at purifying these enzymes have so far failed.

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HEART GLYCOSIDES IN POLIOVIRUS HOST CELL INTERACTION

III. CHEMICAL STRUCTURE AND ACTIVITY

By

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Summary. Digitoxigenin, digitoxin, lanatoside A, digoxin, digoxigenin, lanatoside C and gitoxin at a concentration of 10^{-6} *M*/l reduced the final virion yield and prolonged the lag phase. Compounds having the digitoxigenin base exhibited the greatest absolute activity. At 10^{-7} *M*/l concentration, compounds with digoxigenin base were practically inactive, while those with digitoxigenin base still reduced the yield and prolonged the lag. At concentrations of 10^{-8} *M*/l and lower, all glycosides but gitoxin and both lanatoside A and C caused increase of yield and shortening of lag. Of the aglucons, digoxigenin reduced the yield throughout, whereas digitoxigenin had no effect at dilutions of 10^{-9} *M*/l and higher.

It has been suggested that the extensive yield reduction caused by heart glycosides at high concentrations is the result of a nonspecific coating effect and of specific inhibition of digitalis sensitive $K^+Na^+Mg^{++}$ ATPase. The activity of the compounds appeared to depend on the structure of the steroid part and decreased in the sequence digitoxin > lanatoside A > > digitoxigenin > digoxin = gitoxin > digoxigenin > lanatoside C.

At concentrations of 10^{-8} *M*/l and lower, the activity depended on the molecule's sugar substituent. Lanatosides and glycosides increased the yield similarly, while aglucons reduced it.

A tentative proposal is given on the possible correlations between the conformational changes of ATPase molecules and those of the virion specific receptor sites in the cell membrane.

In previous papers [1, 2] we have reported on the effect of digitoxin and digoxin and their aglucons on the cycle of poliovirus in PMK III/1 permanent monkey kidney cell line. The main conclusion was that these substances were, in a given time and concentration range, effective stimulators or inhibitors of specific virion attachment to the receptors, and/or of the process of eclipse. The effect appeared to depend also on differences in the molecular structure. This paper reports on some further studies along this line.

Materials and methods

Cells and virus, as well as the methods of their cultivation and maintenance have been described earlier [3] and so were the procedures applied for virus titration and one-step growth experiments.

The heart glycosides used in this study were all products of the G. Richter Pharmaceutical Co., Budapest.

Experimental

The structure of the compounds used in this study is shown in Fig. 1.

All compounds have the same perhydro-1,2-cyclopenteno-phenanthrene skeleton and show the structural features of 14-iso-ethiocholane, carrying the following common substituents: 3β and 14β hydroxyl; $17\beta\Delta^{\alpha,\beta}$-$\gamma$-oxy-butenic acid lactone; 10β and 13β methyl groups. The anellation of C/D rings is cis. Digoxigenin and gitoxigenin bear one additional —OH group in 12β and 16β position, respectively. The glycosides and lanatosides have -(digitoxose)$_3$ and -(digitoxose)$_2$-acetyldigitoxose-D-glucose substituents, respectively, in 3β position. Thus regardless whether aglucons, glycosides or

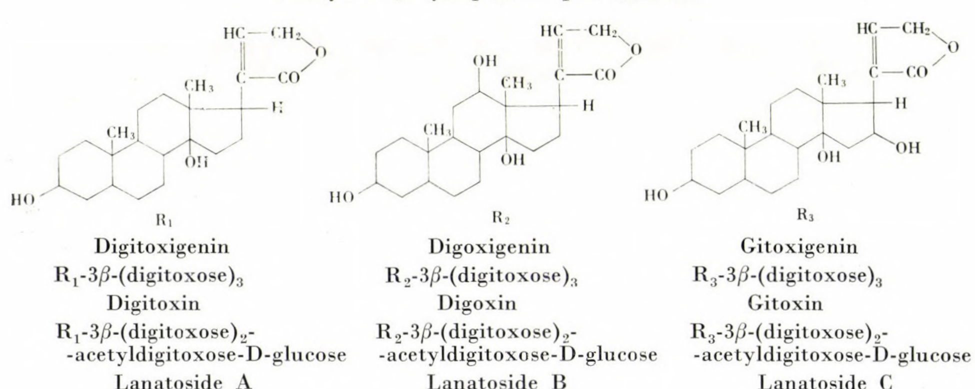

Fig. 1. Digitalis derivatives applied

lanatosides, the molecules differ only in the presence or absence of an additional OH group in 12β or 16β position. The 16β OH group has a negative inductive effect reducing the alkalinity of the carbonyl group in the lactone ring, thus impairing the latter's specific fixation by the active site of ATPase[4].

Results of comparison of dose-response curves of genins and their substituted derivatives are shown in Fig. 2A, B. The individual points represent final virion yields as determined in the 6th hour of the cycle.

It appeared that the overall final virion yield reducing effect at 10^{-6} and 10^{-7} M/l concentrations was higher with digitoxigenin and its derivatives than with digoxigenin and its derivatives. In the presence at 10^{-8} M/l concentration of glycosides and lanatosides of both aglucons the yield increased, while digoxigenin and digitoxigenin exhibited increased and lasting yield reducing activities, respectively. On further dilution of the active substances

a parallel, slow decrease of effectiveness took place, though without any change in the type of the effect produced.

In Fig. 3 are presented the dose-response curves obtained with digitoxin, digoxin and gitoxin. Gitoxin was found to have lost its activity at concentrations of 10^{-8} M/l or less. At 10^{-6} and 10^{-7} M/l concentrations it reduced the yield like digoxin.

The relations between structure and activity are demonstrated in Table I. This contains numerical data on intravenous molar toxicity for the cat [5, 6], the relative arresting doses for the isolated chick embryo heart [7], and the

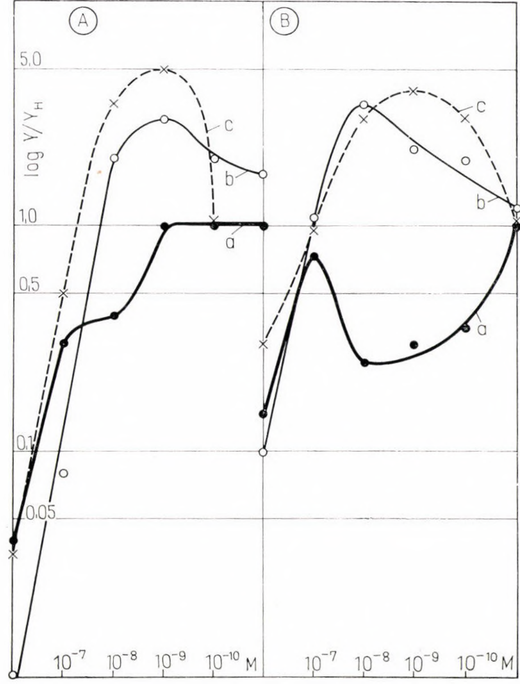

Fig. 2. Dose-response curves with digitoxigenin, digoxigenin, their glycosides and lanatosides. Y means yield in the presence, while Y_H in the absence, of the drug. *A.* a: digitoxigenin; b: digitoxin; c: lanatoside A; *B.* a: digoxigenin; b: digoxin; c: lanatoside C

virion yield reducing activity expressed as the factor of reduction as referred to the drug free control as unit. Reference is made to the compounds' affinity to serum albumin [8], which may considerably affect the concentrations required to obtain the desired effect in intact animals.

At 10^{-6} and 10^{-7} M/l concentrations, the relations of virion yield reducing activity and chemical structure were very similar to those observed with the relative heart arresting doses. The group of compounds with digi-

toxigenin base exhibited the greatest absolute activity. In every group, the glycosides showed the greatest relative activity, followed in succession by the lanatosides and aglucons. Thus at high concentrations the main determinant of activity appeared to be the steroid part of the molecule, while the presence and nature of the sugar component had a secondary importance.

At concentrations of 10^{-8} M/l or lower, all glycosides and lanatosides had increased the final virion yield 2- to 5-fold, apparently independently of the characteristics of the steroid part of the molecule. Gitoxin was an exception as it failed to have any effect in this dilution range.

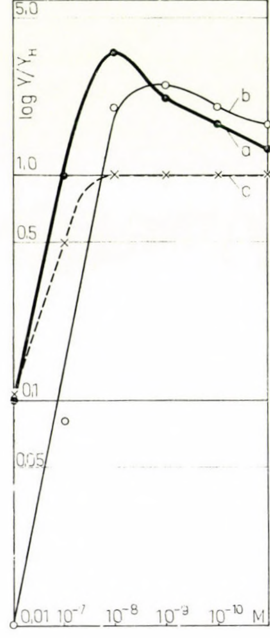

Fig. 3. Dose-response curves with three glycosides. Y means yield in the presence, while Y_H in the absence of the drug. a: digoxin; b: digitoxin; c: gitoxin

Both aglucons tested had maintained a yield reducing type of effect at a concentration of 10^{-8} M/l. These compounds, unlike their sugar substituted derivatives, differed remarkably in effectiveness, depending on their structure. The effect of digoxigenin decreased by factors of 1.1 to 1.3 on further tenfold dilution, while that of digitoxigenin disappeared at a 10^{-9} M/l final dilution. Apparently, the presence of the 12β $-$OH substituent rendered the yield reducing effect of digoxigenin more marked as compared to digitoxigenin lacking this substituent.

Table I

Comparison of some effects of digitalis derivatives

Compound	Reduction of activity by serum albumin [8]	Molar toxicity* for the cat	Relative embryo-heart** arresting dose	Relative yield reducing effect*** at concentration of				
				10^{-6} M/l	10^{-7} M/l	10^{-8} M/l	10^{-9} M/l	10^{-10} M/l
Lanatoside A	5—10-fold	2.56	1.00	28.60	2.00	0.28	0.20	1
Digitoxin		2.18	1.00	100.00	12.50	0.50	0.33	0.50
Digitoxigenin		0.88	8.91	25.00	3.33	2.50	1	1
Lanatoside C	3—5-fold	3.51	4.46	3.33	1.11	0.33	0.25	0.33
Digoxin		2.80	3.08	10.00	1	0.28	0.45	0.55
Digoxigenin		1.55	36.04	6.66	1.33	4.00	3.00	2.85
Gitoxin	Slight if any	1.07	2.83	10.00	2.00	1	1	1

* kg cat killed by the intravenous infusion of 10^{-6} M/l drug (Stoll [6]).
** Figures calculated from the data by De Graff *et al.* [7].
*** Ratio of Y_H/Y, where Y_H is the yield in HBS and Y is that in the presence of the drug. Value 1 means a lack of effect. Numbers in brackets are references.

Discussion

Portius and Repke [4] have undertaken detailed studies on the relations of the structural characters to (K^+ Na^+ Mg^{++}) dependent membrane ATPase inhibitory activity of various digitalis derivatives, and arrived at the following conclusions: (i) The "effective grouping" specifically reacting with the enzyme's active site is the carbonyl group conjugated with the C=C double bond in the lactone. Any modification of this structure results in a considerable decrease or complete loss of inhibitory activity. The presence of a hydroxyl substituent at either C-17 or C-16 will also reduce activity by a negative inductive effect. (ii) The steroid part of the molecule plays the role of a "fixing group", ensuring an irreversible interaction between the effective grouping and the active site. This stabilizing step consists of the formation of short range apolar interactions between the steroid part and the complementary surface of the enzyme. The number, position and nature of polar or apolar substituents of the steroid may either facilitate, limit or inhibit stabilization. (iii) The sugar component bound to the steroid part at C-3 represents the "supporting group". This group being highly polar and flexible relative to the other parts of the molecule, would reduce the chance of formation of nonspecific apolar bindings, thus increasing the specificity of interaction.

As to the mechanism of specific inhibition of (K^+ Na^+ Mg^{++}) dependent ATPase activity, several theories have been proposed. The more recent ones

[9, 10], though different in some details, agree in suggesting the occurrence of alternating conformational changes parallel to the performance of their transfer functions. The inhibitory action of heart glycosides consists essentially of an irreversible "locking" of the enzyme in one of its possible conformations.

Considering our findings in this light, the above principles appeared to offer certain explanations for the effects observed. All compounds used by us possessed the $\Delta^{\alpha,\beta}$-γ-oxy-butenic acid lactone group in 17β position. Thus, in principle, any of them was able to react with the enzyme's active site. At high concentrations all types of compounds tested were found to reduce final virion yield. This effect, however, did not seem to be entirely specific, since these compounds, if present in excess amounts, are known to have a tendency of coating the cells [11]. This fact *per se* is suitable for explaining the uniformly inhibitory character of the effect of all compounds at 10^{-6} and 10^{-7} *M*/l concentrations. The outstanding activity of the compounds with digitoxigenin base may be ascribed to their simultaneous coating and extensive, irreversible, highly specific ATPase-inhibitory effects [12]. The other types of compounds seemed preferentially to undergo mutual homologous intermolecular interactions to form a coat instead of interacting with the ATPase sites to which they were but inadequately complementary.

Concentrations of 10^{-8} *M*/l or less did not permit coat formation owing to quantitative reasons [1], but allowed for free, specific interactions with the specific receptors in the cell membrane. At 10^{-8} *M*/l or ower concentrations, gitoxin, the compound having the least favourable structure to interact with the ATPase [4], failed to influence the final yield in either way. Digitoxin, digoxin as well as lanatosides A and C induced an increase of the final virion yield and a shortening of the lag phase. All these compounds possess the substituent called by PORTIUS and REPKE [4] the "supporting group", which improves the specificity of interaction with ATPase, thus diminishing the unfavourable effect of the extra-OH group in the molecules with digoxigenin base.

From the following considerations we exclude the effects observed at high concentrations of heart glycosides which are known to coat the cells and cause inhibition "mechanically" [11]. Irreversible attachment is supposed to take place through subsequent short range interactions between 1, 2 and 3 virion vertices and cell membrane's receptor sites. The membrane of a freely floating cultured cell is considered a structure in the state of continual transition between its possible metastable states [13]. The individual structural components themselves participate in the above transitions by consistently undergoing conformational changes and appropriate mutual interactions. This process would allow the alternating appearance and disappearance of specifically structured adequate receptor sites. The time required for the specific irreversible attachment of virions would be determined by the prob-

ability of a successive formation of appropriately located 1, 2 and finally 3, receptor sites and by the decrease of freedom of choice parallel to the development of 1, 2 and 3 vertex-site hits. Induced conformational changes of neighbouring structural elements, like "locking" the ATPase in one of its possible conformations by digitalis glycosides may increase or decrease the above outlined probabilities, thus either improve or impair the process of the virion's attachment to, and penetration into, the cell. The "efficiency of infection" [3] may thus be increased or decreased by induced changes in certain non-receptor structural components of the cell membrane. Stabilization of the ATPase in a conformation determined by its interaction with a specific glycoside or lanatoside is supposed to induce stabilization of a properly arranged set of receptors directly facilitating the attachment and indirectly also the penetration, of virions. This would explain both the shortening of the lag phase and the increase of final virion yield [1, 2]. The reaction with the respective aglucons is less specific [4] and also the molecules have a smaller complementary surface as compared to that of glycosides and lanatosides. We therefore suppose the aglucons to cause stabilization of some inappropriate conformation of the ATPase which would again immobilize some, most, or all, neighbouring receptor sites in a conformation not recognizable for the virion vertex. Depending on the incidence of unfavourably affected sites, attachment may still occur through 1 or 2 vertices, while the probability of a 3 vertices — 3 sites attachment is extremely reduced.

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MICROBIOLOGICAL DECOMPOSITION OF 17α-METHYL-17β-HYDROXY STEROIDS WITH ANDROSTANE NUCLEUS

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Summary. Two well known steroid transforming microorganisms, *Nocardia restrictus* (ATCC 14887) and *Mycobacterium phlei* were compared as to their ability to metabolize 17α-methyl-17β-hydroxy steroids with androstane nucleus; they were found to decompose this group of compounds in the same way as the 17-ketosteroids. While in *Nocardia restrictus* the sequence of 9α-hydroxylation and Δ^1-dehydrogenation of the compounds can be interchanged depending on the circumstances; in *Mycobacterium phlei*, formation of the Δ^1 double bond invariably takes place first. The 9α-hydroxylase of both microorganisms is inhibited by 8-hydroxyquinoline. In the presence of that chelating agent, 17α-methyl-17β-hydroxy-androsta-1,4-dien-3-one accumulated in the fermentation broth from substrates of 5α-saturated-3β-hydroxy,5α-saturated-3-keto, Δ^1-5 α-saturated-3-keto, Δ^4-3-keto, Δ^5-3 β-hydroxy and $\Delta^{4,6}$-3-keto structure.

In the present experiments additional information was sought on the steroid transforming ability of *Nocardia restrictus* and *Mycobacterium phlei.* Both microorganisms are known to form a $\Delta^{1,4}$-3-keto structure in the ring A of 17-ketosteroids and to introduce a hydroxyl group in 9α position, resulting in spontaneous cleavage of the ring B with a reverse aldol reaction [1, 2].

As a result of experiments aiming selective side chain cleavage of sterols it turned out that certain chelating agents inhibit the 9 α-hydroxylation in the mentioned as well as in other microorganisms [1, 3, 4, 5].

In the present studies, transformation products obtainable with both microorganisms from 17α-methyl-17β-hydroxysteroids in the presence or absence of 8-hydroxyquinoline were examined. The results allowed conclusions concerning the metabolic pathways. The group of the examined compounds included the pronouncedly androgenic 17α-methyl-17β-hydroxyandrost-4-en-3-one (IV, methyltestosterone) and the anabolic 17α-methyl-17β-hydroxy-androsta-1,4-dien-3-one (VII, Nerobol®).

Materials and methods

Methods of cultivation and microbiological transformation. The *Mycobacterium phid* (deposited under No. 29/1965 at the National Institute of Public Health, Budapest) anle *Nocardia restrictus* ATCC 14887 strains were maintained on potato glucose agar medium. In the experiments both strains were propagated on the following medium: corn-steep liquor, 10 g (dry weight); glycerol, 10 g; tap water to 1000 ml, neutralized with sodium hydroxide, adjusted to pH 6.8—7.2, passed through filter paper, distributed in 100 ml amounts into 500 ml Erlenmeyer flasks and autoclaved at 121 °C for 20 minutes.

Microbiological transformation of steroids was carried out partly with washed cell suspensions, partly with growing cultures. The washed cell suspension was prepared as follows: cells grown on agar slant were suspended in broth. One ml of this suspension was inoculated into growth medium and after being shaken for 48 hours the culture was harvested and resuspended in 0.4% sodium chloride solution. The dry weight of the cell suspension was 6 mg/ml with *M. phlei* and 4 mg/ml with *N. restrictus*. For transformation with growing culture, 6 ml of the shaken culture was inoculated into a flask of the same medium and at the same time the solution of the substrate in N,N'-dimethylformamide, sterilized by passing through a G5 glass filter, was added.

In each case, incubation was carried out on a rotary shaker (300 r.p.m., 2 cm amplitude) in 500 ml flasks each containing 100 ml of culture. Incubation temperature was 37 °C for *M. phlei* and 28 °C for *N. restrictus*. The applied substrate concentrations and incubation periods are specified separately with each compound.

Analytical methods. Transformation was followed up by thin-layer chromatography of 1,2-dichloroethane extracts from samples taken at given intervals from the fermentation mixture (developing solvent, 3% ethanol + 97% chloroform; carrier, silica gel G, Merck).

The 17α-methyl-17β-hydroxyandrosta-1,4-dien-3-one (VII) content of the fermentation mixtures was determined by the simplified thiosemicarbazone (TSC) method [6, 7]. Ten ml of the fermentation broth was extracted with 20 ml of chloroform. Two ml of the organic phase was dried with sodium sulphate and evaporated. The dry residue was dissolved in 2 ml of ethanol, then 4 ml of 0.5 *M* thiosemicarbazide in 0.5 *N* HCl solution was added and the reaction mixture was kept at 40 °C for 30 minutes. Finally the material was diluted by addition of 20 ml ethanol. Extinction was read at $\lambda = 325$ nm wavelength against a simultaneously prepared blind solution. The concentration of 17α-methyl-17β-hydroxyandrosta-1,4-dien-3-one (VII) was calculated from a standard curve.

The melting point was determined in a micro Kofler apparatus. The ultraviolet spectra were taken in a UNICAM SP 500 spectrophotometer, while the infrared spectra in a UR-10 (Zeiss) apparatus.

Transformation of 17α methyl-17β-hydroxy compounds with androstane nucleus in the presence of 8-hydroxyquinoline. Each substrate (I—VI, see Table I) was dissolved in 1 ml of acetone and added to 100 ml of cell suspension of both strains together with the 8-hydroxyquinoline, so as to make final concentrations of 200 μg/ml and 100 μg/ml, respectively, in the fermentation broth. During incubation for 24 hours, transformation was followed up by thin-layer chromatography and finally the 17α-methyl-17β-hydroxy-androsta-1,4-dien-3-one (VII) content of the fermentation broth was assessed quantitatively by the TSC method.

Isolation and structural identification of 17α-methyl-3,17β-dihydroxy-9,10-secoandrosta-1,3,5(10)-trien-9-one (VIII). To 1 litre of washed *M. phlei* cell suspension 2 g of compound VII, dissolved in 20 ml of dimethylformamide was added and incubated for 20 hours. Subsequently the cells were removed by filtration, the filtrate was extracted with 3 × 200 ml of 1,2-dichloroethane and the combined extract was dried on Na_2SO_4 and evaporated. The dry residue was decolorized by charcoal in methanol, then crystallized twice from benzene. Finally 123 mg of VIII was yielded. Melting point: 166—167 °C. $[\alpha]_D$ —10° (c = 1, 95% ethanol). Analysis: Calculated for $C_{20}H_{28}O_3$ (316.42): C, 75.91; H, 8.92; found: C, 75.97; H, 8.97. λ_{max} in ethanol 280 nm (ε 2150). Infrared spectrum (KBr) ν_{OH} 3525, 3600—3100 (max. 3330), $\nu_{C=O}$ 1710, aromatic bands 3030, 1615—1595, 1510—1470, 870, 815 cm^{-1}.

To verify the structure, 250 mg of compound VIII was acetylated in 1 ml of absolute pyridine with 1 ml of acetic anhydride for 16 hours at room temperature. Crystallizing the crude product from diisopropylether, 160 mg of 17α-methyl-3,17β-dihydroxy-9,10-secoandrosta-1,3,5(10)-trien-9-one 3-acetate was obtained. Melting point: 106—108 °C, $[\alpha]_D$ —7° (c = 1, chloroform). Analysis: Calculated for $C_{22}H_{30}O_4$ (358.46): C, 73.71; H, 8.44; found: C, 73.51; H, 8.63. λ_{max} in ethanol 265 nm (ε 603), 273 nm (ε 580), infrared spectrum (KBr) ν_{OH} 3500—3200 (max. 3370), $\nu_{C=O}$ (phenolester) 1770; $\nu_{C=O}$ (ketone) 1710, aromatic bands 3030, 1610, 1590, 1505, 1470, 835, 815, ν_{C-O} (ester band) 1218, 1200, 1020 cm^{-1}.

Isolation and structural identification of 17α-methyl-9α,17β-dihydroxyandrost-4-en-3-one (IX). Two g of IV was added to 400 ml of growing *N. restrictus* culture. After incubation for 12 hours, the fermentation broth was extracted with 3 × 100 ml of n-butanol. The combined extract was evaporated and the dry residue (2.3 g) dissolved in 100 ml of chloroform. This was washed with 2 × 50 ml of 5% $NaHCO_3$, subsequently with 4 × 50 ml of 4% NaOH. The sodium hydroxide extract was neutralized with acetic acid. The precipitated product was filtered, and after recrystallizing it twice from benzene, 61 mg of compound VIII was obtained. The organic phase, extracted with alkali, was neutralized by washing with water and evaporated. The dry residue (860 mg) which according to thin-layer chromatography contained compounds IV and IX, was fractionated on Florisil column (80 g of Florisil suspended in 1,2-dichlo-

roethane). Elution was carried out with 1,2-dichloroethane solvent mixtures containing increasing amounts of acetone. IX was obtained by elution with 10% acetone containing 1,2-dichloroethane solvent. From the evaporation residues of the fractions which contained IX, 88 mg of this was obtained on recrystallization from methanol and ether. Melting point: 192—193 °C, $[\alpha]_D$ 76° (c = 1, chloroform). Analysis: Calculated for $C_{20}H_{30}O_3$ (318.44): C, 75.43; H, 9.50; found: C, 75.71; H, 9.69. λ_{max} ethanol 242 nm (ε = 15 300). Infrared spectrum ν_{OH} 3585, 3600—3200 (max. 3480), $\nu_{=CH}$ 3040, $\nu_{C=O}$ 1665, $\nu_{C=C}$ 1620 cm^{-1}.

Attempts were made to acetylate IX. Thirty mg of it was dissolved in 1 ml of absolute pyridine, then 0.5 ml of acetic anhydride was added and the reaction mixture was allowed to stand at room temperature for 48 hours and subsequently diluted with 30 ml of water. This solution was extracted with 3 × 10 ml of ethyl acetate and the extract was dried by evaporation. On crystallization of the residue from methanol—ether, IX was obtained. On analysis by thin-layer chromatography of the mother liquor no other steroid than IX was detectable.

Attempts were made to oxidate the new hydroxyl group formed during fermentation. Thirty mg of IX was dissolved in 1 ml of acetic acid and 40 mg of CrO_3 dissolved in a mixture of 0.25 ml water and 0.25 ml acetic acid was added. The reaction mixture was allowed to stand at room temperature for 4 hours. After processing in the same way as described for acetylation, only IX was obtained.

Transformation of 17α-methyl-9α,17β-dihydroxyandrost-4-en-3-one (IX) to 17α-methyl-3,17β-dihydroxy-9,10-secoandrosta-1,3,5(10)-trien-9-one (VIII). A washed cell suspension of *Nocardia restrictus* was diluted tenfold with 0.4% sodium chloride solution. To the culture diluted to 2 litres, 200 mg of IX, dissolved in 20 ml of acetone, was added. Fermentation was allowed to take place for 6 hours, then the culture was processed in the same way as described in the paragraph on isolation of IX. An 11 mg amount of VIII was obtained, whose IR and UV spectra and melting point were identical to the constants already established. Using *M. phlei*, the same fermentation and processing methods yielded 8 mg of VIII; in that particular case, owing to the culture's weaker activity, a 5-fold dilution was only prepared.

Results

In the presence of 8-hydroxyquinoline, both *Mycobacterium phlei* and *Nocardia restrictus* produced 17α-methyl-17β-hydroxyandrosta-1,4-dien-3-one (VII) from steroids having 5α saturated-3β-hydroxy, 5α-saturated-3-keto-, Δ^1-5α-saturated-3-keto, Δ^4-3-keto, Δ^5-3β-hydroxy and $\Delta^{4,6}$-3-keto structure

Table I

Production of 17α-methyl-17β-hydroxyandrosta-1,4-dien-3-one (VII) from different substrates

Substrate	Compound VII formed in 24 hours (mole per cent)	
	M. phlei	*N. restrictus*
17α-methyl-5α-androstane-3,17β-diol (I)	49	76
17α-methyl-17β-hydroxy-5α-androstan-3-one (II)	51	82
17α-methyl-17β-hydroxy-5α-androst-1-en-3-one (III)	71	80
17α-methyl-17β-hydroxyandrost-4-en-3-one (IV)	74	82
17α-methylandrost-5-ene-3β,17β-diol (V)	74	64
17α-methyl-17β-hydroxyandrosta-4,6-dien-3-one (VI)	73	64

200 μg/ml of substrate was transformed with washed cells in the presence of 100 μg/ml of 8-hydroxyquinoline. The product was estimated with the TSC method.

on the 17α-methyl-17β-hydroxyandrostane nucleus. The transforming abilities of the two strains as determined in a typical experiment are shown in Table I.

At the end of fermentation, the mixtures contained compounds other than VII only in traces, except for the transformation of I and II with *Mycobacterium* phlei, when 10% of II was demonstrable by thin-layer chromatography.

If during fermentation no 8-hydroxyquinoline was added to the culture, decomposition of the steroid nucleus took place. On fermentation of 17α-methyl-17β-hydroxyandrosta-1,4-dien-3-one (VII) with *Mycobacterium phlei*, a new compound having the empirical formula $C_{20}H_{28}O_3$ was obtained. Its solubility in alkali as well as its ultraviolet spectrum suggested a phenol type. Its infrared spectrum showed hydroxyl bands, a ketone band of a six-membered ring and bands characteristic of an aromatic ring. Of the latter bands those appearing at 870 and 815 cm^{-1} indicated the possibility of a 1,2,4-trisubstitution of the aromatic ring.

Accordingly, it seems that similarly to the decomposition of androsta-1,4-diene-3,17-dione, VII also gives rise to 9,10-secophenol after 9α-hydroxylation and subsequent spontaneous reverse aldol reaction. The above data of the new compound correlate well with the expectation of a 17α-methyl-3,17β-dihydroxy-9,10-secoandrosta-1,3,5(10)-trien-9-one structure (VIII). The new compound was acetylated with acetic anhydride in pyridine at room temperature. The infrared spectrum of the obtained product showed bands typical of the phenol-ester group and also a hydroxyl band; thus, the phenolic hydroxyl group became acetylated, while the tertiary hydroxyl on carbon 17 remained free — just as expected — under the applied mild conditions of acetylation.

VIII was also obtained on transformation of 17α-methyl-9α,17β-dihydroxyandrost-4-en-3-one (IX) with *Mycobacterium phlei.* In that case, the bacterial culture had to produce Δ^1-dehydrogenation in order that secophenol (VIII) be formed through spontaneous reverse aldol reaction. But we were not able to discover any appropriate condition to transform 17α-methyl-17β-hydroxyandrost-4-en-3-one (IV) to 17α-methyl-9α,17β-dihydroxyandrost-4-en-3-one (IX) by *M. phlei.*

17α-Methyl-9α,17β-dihydroxyandrost-4-en-3-one (IX) was obtained from IV with a growing culture of *N. restrictus.* (Under such conditions, both microorganisms transformed androst-4-ene-3,17-dione into its 9α-hydroxyl derivative [1, 8].) During fermentation VIII was also formed, implying that two subsequent intermediary compounds of the transformation process had accumulated. Under different conditions, when IX was fermented with resting (washed) cells of *N. restrictus*, VIII was obtained, like under similar circumstances with *M. phlei.* 17α-Methyl-9α,17β-dihydroxyandrost-4-en-3-one (IX) has been described in the literature [9], and the melting point of the compound

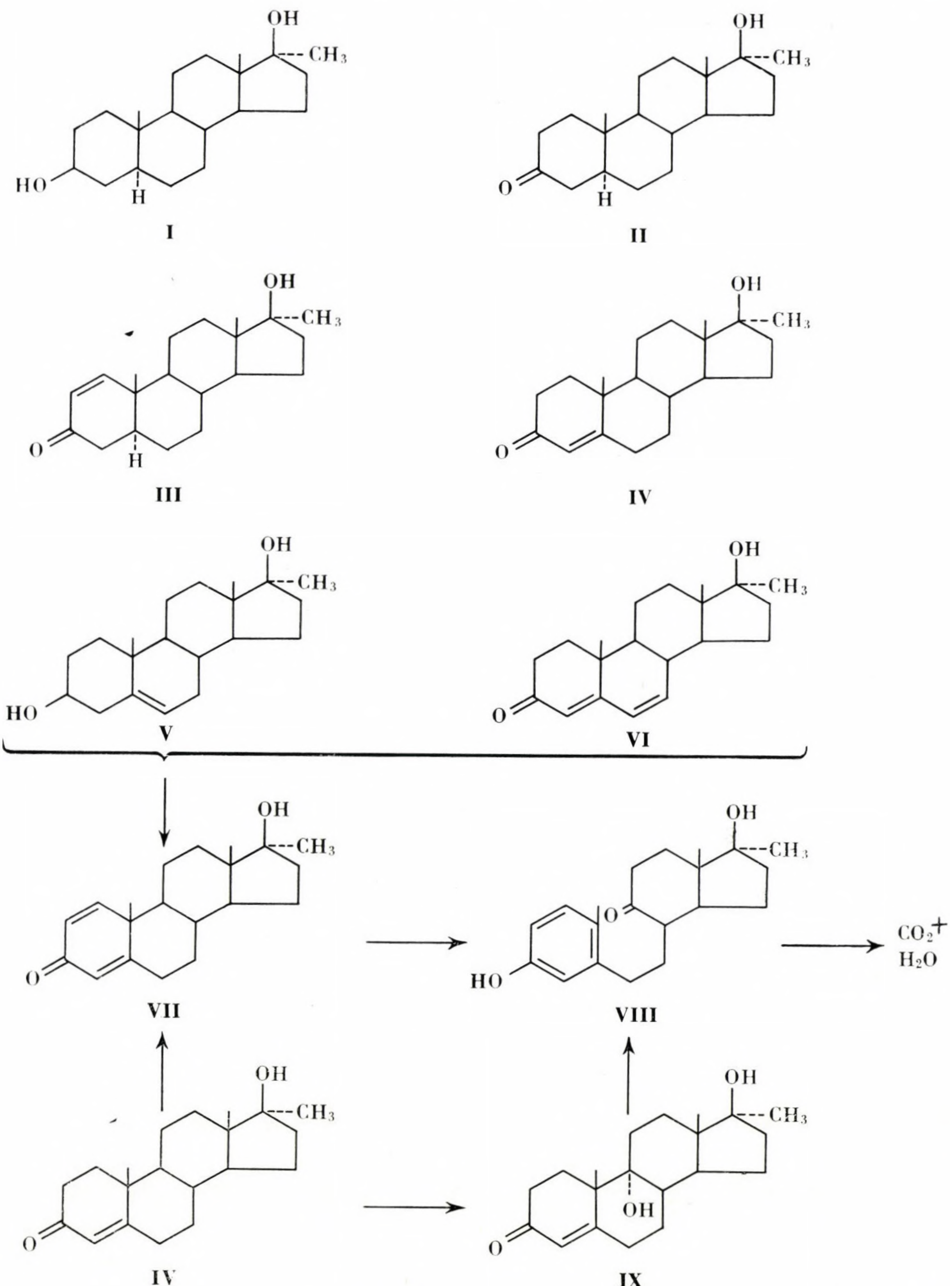

Fig. 1. Scheme suggested for bacterial transformations in the methyltestosterone series. Conversions leading to VII are well observable in the presence of a chelating agent. The step from IV to IX (bottom) was not found in *M. phlei.* I: 17α-methyl-5α-androstane-3β,17β-diol, II: 17α-methyl-17β-hydroxy-5α-androstan-3-one, III: 17α-methyl-17β-hydroxy-5α-androst-1-en-3-one, IV: 17α-methyl-17β-hydroxy-androst-4-en-3-one, V: 17α-methylandrost-5-ene-3β,17β-diol, VI: 17α-methyl-17β-hydroxyandrosta-4,6-dien-3-one, VII: 17α-methyl-17β-hydroxyandrosta-1,4-dien-3-one, VIII: 17α-methyl-3,17β-dihydroxy-9,10-secoandrosta-1,3,5(10)-trien-9-one, IX: 17α-methyl-9α,17β-dihydroxy-androst-4-en-3-one

isolated by us corresponded to the reported one, but no data have been published on its optical rotation. Elementary analysis of the substance correlated well with the calculated data for the empirical formula. As expected, the hydroxyl group incorporated during fermentation was tertiary, *i.e.* it resisted acetylation with acetic anhydride in pyridine at room temperature as well as oxidation with chromic acid in aqueous acetic acid medium. Under such conditions, both primary and secondary hydroxyl groups would have undergone acetylation or oxidation.

Discussion

Compounds with 17α-methyl-17β-hydroxyandrostane nucleus seem to be appropriate substrates for the steroid transforming enzyme systems of both *Nocardia restrictus* and *Mycobacterium phlei.* With both strains, there was evidence of Δ^1- and Δ^4-dehydrogenase, $\Delta^5 \rightarrow \Delta^4$ isomerase and 3β-hydroxydehydrogenase activity as well as of reduction of the Δ^6 double bond. Observation of these functions was facilitated by the fact that 9α-hydroxylation as the primary step of decomposition of the ring structure was inhibited by 8-hydroxyquinoline.

Cleavage of the steroid nucleus took place to the analogy of 17-ketosteroids between carbons 9 and 10, resulting in the formation of 17α-methyl-3,17β-dihydroxy-9,10-secoandrosta-1,3,5(10)-trien-9-one (VIII). In principle, the formation of this compound, *i.e.* cleavage of the ring, may take place in two ways depending on whether hydroxylation of C-9 or dehydrogenation at C-1, C-2 occurs first. In the first case the intermediary compound will be 17α-methyl-9α,17β-dihydroxyandrost-4-en-3-one (IX), whereas in the second, 17α-methyl-17β-hydroxyandrosta-1,4-dien-3-one (VII).

In the case of *N. restrictus*, both metabolic pathways have been confirmed by altering the conditions of fermentation, as both intermediary compounds have been isolated. With *M. phlei*, only the intermediary compound VII appeared in addition to VIII, in circumstances under which *N. restrictus* produced IX. It seems that for the 9α-hydroxylase of *M. phlei*, 17α-methyl-17β-hydroxyandrost-4-en-3-one (IV) is an inappropriate substrate, while 17α-methyl-17β-hydroxyandrosta-1,4-dien-3-one (VII) is an adequate one.

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TRANSFORMATION OF 4,5-EPOXYSTEROIDS WITH MYCOBACTERIUM PHLEI

I. TRANSFORMATION UNDER AEROBIC CONDITIONS

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Summary. 4,5-Epoxysteroids containing 17β side chain or 4,5-epoxyandrostanes were transformable with *Mycobacterium phlei* under aerobic conditions to androsta-1,4-diene-3,17-dione. As intermediate androst-4-ene-3,17-dione was found. In the course of transformation a transitory reduction of the 3-oxo group, and dehydrogenation of the 17β-hydroxyl group, if present, was observed.

The formation of epoxysteroids by microorganisms is wellknown [1, 2]. A similar mechanism of oxido group formation by mammalian tissue has been indicated by the few experiments conducted along this line [1, 3]. Metabolisation of the oxido group of steroids under biological conditions has also been studied by several authors, with unequivocal conclusions. Dihydroxy compounds resulting from the cleavage of the oxido group were isolated by several authors, thus Procházka *et al.* obtained from 5ξ,6ξ-epoxy-3β-hydroxy-B-nor-androstan-17-one the appropriate 3β,5α, 6β-trihydroxy derivative with *Rhizopus nigricans* [4]. Camerino and Sciaky [5] isolated 3β,4β,5α-trihydroxypregnan-20-one by means of yeast from 4β,5β-epoxypregnane-3,20-dione, and the corresponding 3β,5α,6β-trihydroxy derivative from 5α,6α-epoxy-3β-hydroxypregnan-20-one. On transformation of 16α,17,α-epoxypregn-4-ene-3,20-dione, with *Cylindrocarpon radicicola* culture El Tayeb *et al.* [6] observed after the decomposition of the side chain the formation of 16α,17β-dihydroxyandrosta-1,4-dien-3-one. As to other ways of transformation of the same substrate, Camerino *et al.* obtained with yeast 17β-methyl-16α,20α-dihydroxy-17α-pregna-4,13-dien-3-one, while Wix and Albrecht [8], using *Fusarium caucasicum*, obtained androsta-1,4-diene-3,17-dione. Kadis [3] demonstrated the formation of 17α-hydroxypregn-4-ene-3,20-dione by swine ovarian tissue, also from 16α,17α-epoxypregn-4-ene-3,20-dione [3].

In the present study, transformation of some 4,5-epoxysteroids by *Mycobacterium phlei* has been examined undeı aerobic conditions.

Materials and methods

Melting point was determined in a Kofler-type apparatus, and the value was uncorrected. Infrared spectrum was recorded by a UR-10 instrument, whereas the NMR spectrum by a JEOL-NMC-60-type spectrophotometer.

Preparation of 4,5-epoxy steroids used for transformation. The 4,5-epoxy-3-oxo compounds were obtained from the corresponding Δ^4-3-oxo compounds in alkaline medium with hydrogen peroxide: 4β,5β-epoxycholestan-3-one (I) [9]; 4β,5β-epoxypregnane-3,20-dione (II) and 4α,5α-epoxypregnane-3,20-dione (III) [10], 4β,5β-epoxyandrostane-3,17-dione (IV) [11], and 4α,5α-epoxyandrostane-3,17-dione (V) [12]. 4β,5β-epoxyandrostane-3α, 17β-diol (VI) was prepared with sodium borohydride from 4β,5β-epoxy-17β-hydroxyandrostan-3-one [13].

Growth of bacterium and microbiological transformation. Mycobacterium phlei strain No. 29/1965, deposited in the National Institute of Public Health, Budapest, was maintained on potato-dextrose agar. From the approximately 1 week old culture a suspension was prepared with 8 ml sterile water and 1 ml amounts of this suspension were inoculated each into 100 ml nutrient medium. The medium consisted of 1% glycerol and 1.5% cornsteep liquor in water. The medium was adjusted to pH 6.5—7.0, then it was filtered, distributed in 100 ml amounts each to 500 ml flasks and sterilized at 121 °C for 15 minutes. The inoculated flasks were incubated in a horizontal shaker of 180/minute periodicity and 30 mm eccentricity, at 37 °C for 48 hours. For transformation, the culture was centrifuged and the sediment was resuspended in sterile distilled water, using for analytical purposes 50 ml amounts in 500 ml Erlenmeyer flasks, whereas for preparative purposes 500 ml amounts in 2000 ml flasks. The cell suspension thus obtained contained 0.5% dry material in a pH range from 5.5 to 6.5; this pH value remained unchanged during transformation even if no buffer was added. The acetonic solutions of steroid substrates were added in a final concentration of 200 μg/ml to the cell suspension. To inhibit the cleavage of the steroid nucleus, 8-hydroxyquinoline at a concentration of 90 μg/ml was added to the suspension [16]. Subsequently, the culturing flasks were incubated further under growth conditions. At the desired time, transformation was completed by filtration and extraction. In experiments performed for analytical purposes, the filtrate was extracted three times with $^1/_4$ volume of dichloroethane, and the cells were washed in dichloroethane. The extract and the washing solvent were combined and washed free of 8-hydroxyquinoline with 2% aqueous oxalic acid solution, desiccated and evaporated to dryness *in vacuo*. The residue was dissolved in acetone and analysed as described below. In the preparative experiments the extract of the filtered fermentation broth was used for isolation.

Analytical methods. Qualitative detection of the transformation products was made by thin-layer chromatography, carried out on 0.25 mm layers of Kieselgel-$PF_{254-366}$ Merck. The solvent system was benzene–ethylacetate 1 : 1 except in the case of 4β,5β-epoxycholestan-3-one when it was 9 : 1. For detection, a spray of sulphuric acid in ethanol 1 : 1 was used, followed by heating at 110 °C. For quantitative determination, androst-4-ene-3,17-dione and androsta-1,4-diene-3,17-dione were estimated by paper chromatography [8], the 4,5-epoxy-steroids were separated by thin-layer chromatography (adsorbent, Kieselgel G, Merck; solvent system, benzene–ethylacetate 1 : 1). The compounds separated by thin-layer chromatography were eluted, evaporated and the residue was dissolved in 3—6 ml of a mixture of 50 ml of 96% ethanol in 70 ml of sulphuric acid kept for 30 minutes at 40 °C, and the extinction was immediately determined with a UNICAM spectrophotometer at a typical wavelength close either to 300 or 500 mμ against blind. Using 10—200 μg of the 4,5-oxido compounds a linear relationship was found between concentration and extinction. The quantity of steroids analysed after transformation was 70—80 $M\%$, as related to the amount of substrate used.

Isolation and identification of transformation products. The known metabolites of the different substrates, such as androst-4-ene-3,17-dione (VII), androsta-1,4-diene-3,17-dione (VIII) as well as 4β,5β-epoxyandrostane-3,17-dione (IV) formed again during transformation were isolated by preparative thin-layer chromatography, carried out on 1 mm layers of Kieselgel G (Merck). Solvent system for the separation of androst-4-ene-3,17-dione and androsta-1,4-diene-3,17-dione were in succession benzene–ethylacetate 19 : 1 and 4 : 1. The solvent for 4,5-oxido compounds was benzene–ethylacetate 1 : 1. The isolated materials were identified by their melting point, mixed melting point and spectroscopic behaviour.

Isolation of 4β,5β-epoxy-3α-hydroxyandrostan-17-one (IX). 1.0 g of 4β,5β-epoxyandrostane-3,17-dione was transformed in the absence of 8-hydroxyquinoline over 4 hours, according to the method for preparative purposes described above. The dry residue obtained on extraction (1.1 g) was dissolved in benzene and chromatographed on 100 g regenerated silica gel [14]. On elution with benzene containing increasing amounts of ethylacetate, first 0.05 g of 4β,5β-epoxyandrostane-3,17-dione (IV), subsequently — at 20% ethylacetate —

0.50 g of 4β,5β-epoxy-3α-hydroxyandrostan-17-one was isolated. After repeated recrystallization from acetone-petrolether the melting point was 183—186 °C (dec). $[\alpha]_D^{25} = +99°$ (c = 1%, acetone). Analysis calculated for $C_{19}H_{23}O_3$ (304.2): C, 74.95; H, 9.27%. Found, C, 74.99; H, 9.39%. IR spectrum: ν_{CO}: 1734 cm^{-1}; ν_{OH}: 3440—3490 cm^{-1}. NMR spectrum: C_4—H: 7.13 ppm (singulette), in $CDCl_3$.

Oxidation of compound (IX). In aqueous acetic acid, compound (IX) with chromic trioxide yielded the corresponding 3-oxo compound (IV).

4α,5α-epoxy-3β-hydroxyandrostan-17-one (X) was isolated on the transformation of 4α, 5α-epoxyandrostane-3,17-dione under anaerobic conditions [15].

Results

The course of transformation of the individual substrates was examined in the experiments for analytical purposes (see Materials and methods).

The data shown in the Table and Figures represent values expressed in *M* per cent of substrate in relation to time.

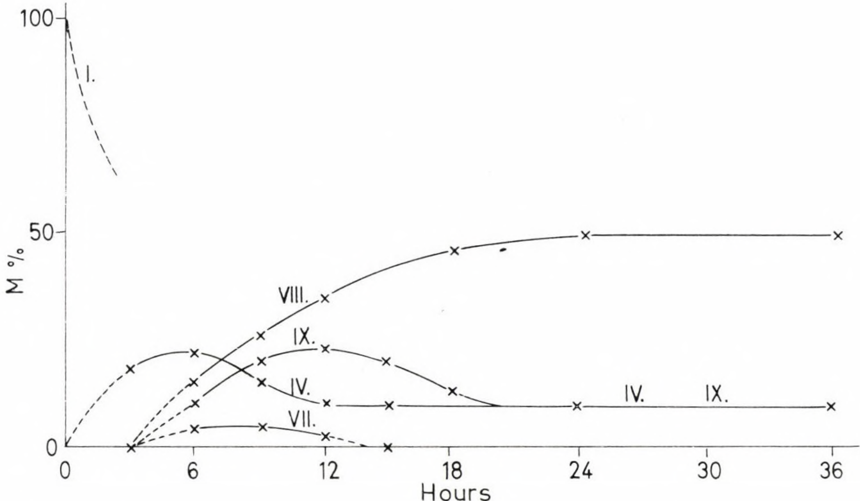

Fig. 1. I = 4β,5β-epoxycholestan-3-one; IV = 4β,5β-epoxyandrostane-3,17-dione; VII = androst-4-ene-3,17-dione; VIII = androsta-1,4-diene-3,17-dione; IX = 4β,5β-epoxy-3α-hydroxyandrostan-3-one

From the 4,5-oxido compounds containing 17β side chain, the transformation of 4β,5β-epoxycholestan-3-one (I), 4β,5β-epoxypregnane-3,20-dione (II) and 4α,5α-epoxypregnane-3,20-dione (III) were examined.

Fig. 1 shows the transformation of 4β,5β-epoxycholestan-3-one in the presence of 8-hydroxyquinoline. Unchanged substrate was still detectable in the 12th hour, but the quantitative assay was unreliable owing to massive contamination with substances of identical R_f value. Apparently, androstane derivatives were present in approximately 50% already in the 6th hour and

subsequently tended to increase until reaching approximately 70% in the 36th hour. In earlier samples, 4β,5β-epoxyandrostane-3,17-dione (IV), later its 3α-hydroxy derivative (IX) showed each a flat maximum. With the transitional appearance of a small amount of androst-4-ene-3,17-dione (VII), 50% androsta-1,4-diene-3,17-dione (VIII) was formed. In different experiments performed under identical conditions the qualitative distribution was the same, as the amount of androsta-1,4-diene-3,17-dione varied between 40 and 55%.

On transforming 4β,5β-epoxypregnane-3,20-dione (II) in the presence of 8-hydroxyquinoline, the qualitative results were the same as with 4β,5β-epoxycholestan-3-one (I). At earlier points of time epoxyandrostanes (IV and IX) amounted to 50%. On transformation of 4α,5α-epoxypregnane-3,20-dione (III), the substrates androst-4-ene-3,17-dione (VII) and androsta-1,4-diene-3,17-dione were found.

In Table I are listed the 36-hour transformation products in the presence of 8-hydroxyquinoline, of 4β,5β-epoxycholestan-3-one (I), 4β,5β-epoxypregnane-3,20-dione (II), 4α,5α-epoxypregnane-3,20-dione (III) and, for comparison, of 4β,5β-epoxyandrostane-3,17-dione (IV). At that point of time no compounds of cholestane or pregnane structure were demonstrable. In every case a greater amount of androsta-1,4-diene-3,17-dione was found from 4β,5β-epoxy-3-oxo compounds with side chain than from 4β,5β-epoxyandrostane-3,17-dione.

From the 4,5-epoxyandrostanes, the transformation of 4β,5β-epoxyandrostane-3,17-dione (IV), 4β,5β-epoxyandrostane-3α,17β-diol (VI) and 4α,5α-epoxyandrostane-3,17-dione (V) was examined.

Fig. 2 shows the transformation of 4β,5β-epoxyandrostane-3,17-dione (IV) in the presence of 8-hydroxyquinoline. The 3-oxo group of the substrate

Table I

Substrate	Transformation products in the 36th hour			
	IV	VII	VIII	IX
I	20	—	50	10
II	22	2—3	42	10
III	—	2—3	70	—
IV	50	—	23	10

I = 4β,5β-epoxycholestan-3-one
II = 4β,5β-epoxypregnane-3,20-dione
III = 4α,5α-epoxypregnane-3,20-dione
IV = 4β,5β-epoxyandrostane-3,17-dione
VII = androst-4-ene-3,17-dione
VIII = androsta-1,4-diene-3,17-dione
IX = 4β,5β-epoxy-3 α-hydroxyandrostan-17-one

obviously underwent a rapid extensive reduction (IX) in the initial phase, then from the 6th hour on, the substrate was formed back again. The qualitative scheme of the formation of androst-4-ene-3,17-dione (VII) and androsta-1,4-diene-3,17-dione (VIII) corresponded to that found on the transformation of $4\beta,5\beta$-epoxycholestan-3-one (Fig. 1). In the experiment in question, 20% androsta-1,4-diene-3,17-dione was demonstrated; in other experiments performed under identical conditions its amount varied between 16 and 28%.

Using $4\beta,5\beta$-epoxyandrostane-3 $\alpha,17\beta$-diol (VI) as substrate, a rapid dehydrogenation of the 17β-hydroxyl group was observed. Transformation

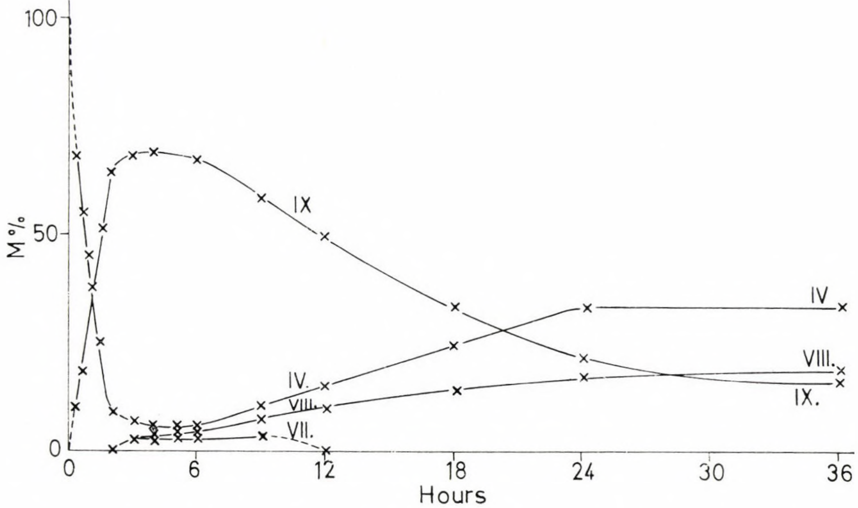

Fig. 2. IV = $4\beta,5\beta$-epoxyandrostane-3,17-dione; VII = androst-4-ene-3,17-dione; VIII = androsta-1,4-diene-3,17-dione; IX = $4\beta,5\beta$-epoxy-3α-hydroxyandrostan-17-one

in the presence of 8-hydroxyquinoline is shown in Fig. 3. After the 2nd hour, exclusively 17-oxo compounds were demonstrable. Changes in the amount of 3α-hydroxy and 3-oxo compounds (IX and IV) were analogous with the transformation shown in Fig. 2: in the early phase the 3α-hydroxy-compound, while later the 3-oxo compound was predominant. According to our findings, transformation of $3\alpha,17\beta$-diol (VI) resulted in a higher level of androsta-1,4-diene-3,17-dione (32—40%) than of $4\beta,5\beta$-epoxyandrostane-3,17-dione (IV), although the same culture was used.

Transformation of $4\alpha,5\alpha$-epoxyandrostane-3,17-dione (V) in the presence of 8-hydroxyquinoline is shown in Fig. 4. In this case, too, there was a reduction of the 3-oxo compound but at least by 10—15% (X). At the 36th hour 70% androsta-1,4-diene-3,17-dione and 5% substrate were measured, while about 5% androst-4-ene-3,17-dione was demonstrable throughout the transformation.

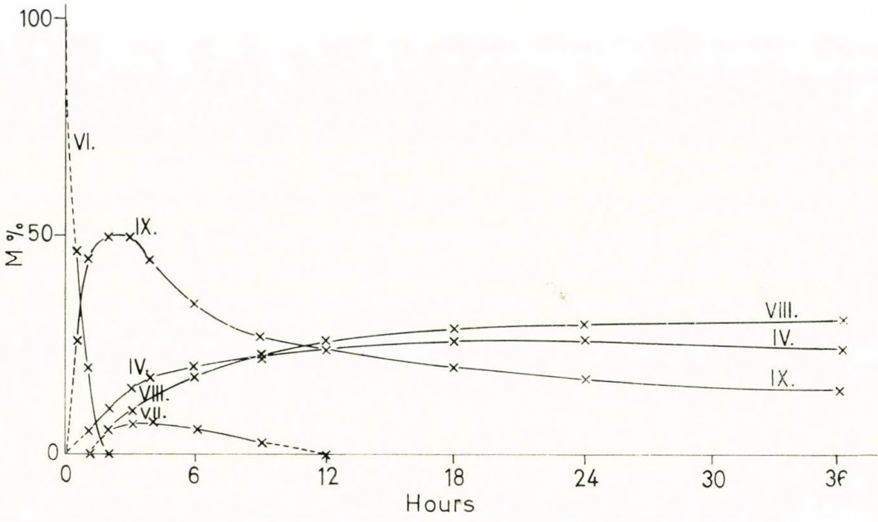

Fig. 3. IV = 4β,5β-epoxyandrostane-3,17-dione; VI = 4β,5β-epoxyandrostane-3α,17β-diol-VII = androst-4-ene-3,17-dione; VIII = androsta-1,4-diene-3,17-dione; IX = 4β,5β-epoxy; 3α-hydroxyandrostan-17-one

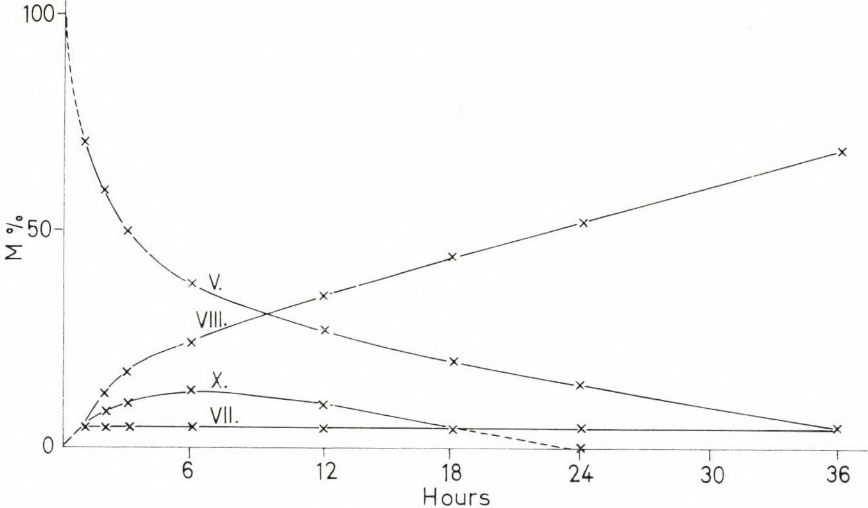

Fig. 4. V = 4α,5α-epoxyandrostane-3,17-dione; VII = androst-4-ene-3,17-dione; VIII = androsta-1,4-diene-3,17-dione; X = 4α,5α-epoxy-3β-hydroxyandrostan-17-one

In every case, 4α,5α-epoxyandrostane-3,17-dione (V) and 4α,5α-epoxypregnane-3,20-dione (III) gave rise to a greater amount of androsta-1,4-diene,3,17-dione, than the corresponding 4β,5β-oxido compounds (IV and II). This greater amount either approximated or attained the quantity of androsta-1,4-diene-3,17-dione obtainable from androst-4-ene-3,17-dione or pregn-4-ene-3,20-dione [16].

Transforming 4,5-oxido compounds (I—IV) in the absence of 8-hydroxyquinoline, a marked decomposition of the steroid nucleus was observed. On transformation of 4β,5β-epoxycholestan-3-one (I), 4β,5β-epoxypregnane-3,20 -dione (II), 4 β,5 β-epoxyandrostane-3,17-dione (IV) and 4β,5β-epoxyandrostane-3α,17β-diol (VI) we could demonstrate androst-4-ene-3,17-dione (VII) only in traces and androsta-1,4-diene-3,17-dione (VIII) in less than 5%, exclusively about the 3rd hour. The substrates differed respectively the rates of degradation. Epoxyandrostanes (IV, IX) formed from 4β,5β-epoxycholestan-3-one (I) and 4β,5β-epoxypregnane-3,20-dione (II) by cleavage of the side chain were detectable up to the 9th—12th hour, but if the substrate was a 4β,5β-epoxyandrostane compound (IV, VI), compounds IV and IX were present at a total concentration of 10—20% up to the 24th hour. On transformation of 4α,5α-epoxypregnane-3,20-dione (III) and 4α,5α-epoxyandrostane-3,17-dione (V), the substrate was demonstrable up to the 6th hour, whereas other steroids could not be demonstrated either after, or before that time.

Discussion

Earlier studies have shown that the *Mycobacterium phlei* strain isolated in our laboratory was capable of metabolizing a wide range of steroids. When transformation was carried out in the presence of a chelating agent such as 8-hydroxyquinoline, there was an accumulation of androsta-1,4-diene-3,17-dione [16, 17], indicating that decomposition of the steroid skeleton [18] was prevented by the inhibition of 9α-hydroxylase [16]. Another way to prevent decomposition of the steroid nucleus was the introduction of a 6β,19-oxido group, indicating that the statement by SIH *et al.* [19] was valid for our strain [20].

It is known that in most cases, formation of the Δ^4 double bond precedes the 1,2- dehydrogenation [21] and the subsequent decomposition of the steroid nucleus. We supposed that the introduction of a 4,5-oxido group would inhibit the formation of a Δ^4, *viz.* Δ^1 double bond and accordingly also the rupture of the steroid skeleton, yet the breakdown of the 17β side chain would nevertheless take place.

Our results clearly indicate that the oxido group had actually not interfered with the breakdown of the side chain; epoxyandrostanes were demonstrated at low concentration (IV, IX). On the other hand, it influenced the rate of cleavage of the nucleus in accordance with its steric position, although the rate was fairly high even in the case of the slowly metabolized 4β,5β-epoxyandrostane-3,17-dione. As in the course of transformation, androst-4-ene-3,17-dione, or androsta-1,4-diene-3,17-dione were temporarily demonstrable, the disappearance of steroids is explained by the known de-

Fig. 5. Tentative sequence of transformational steps

composition of the latter compound, which in further experiments was prevented by 8-hydroxyquinoline [16].

On transformation in the presence of 8-hydroxyquinoline, in the 36th hour a 70—80% amount of steroid metabolites was measured in relation to the amount of substrate. The rate of the process decreased, enabling the estimation of the tentative sequence of the individual steps of transformation, as shown in Fig. 5.

During the transformation of compounds with 17β side chain (I, II), 30—50% of 4β5,β-epoxyandrostane derivatives (IV, IX) were demonstrable, but Δ^4 or $\Delta^{1,4}$ steroids containing side chain were not found. Fig. 1 shows that the formation of 4β,5β-epoxyandrostane-3,17-dione (IV) had preceded that of an appropriate 3α-hydroxy compound (IX), and since no side chain containing 3-hydroxy derivative was demonstrable, the tentative sequence of transformation steps shown in Fig. 5 seemed to be partly proved. On transformation of 4α,5α-epoxypregnane-3,20-dione (III), compounds VII and VIII were only demonstrable in addition to the substrate. In that particular case, intermediary epoxyandrostanes (V, X) were only postulated.

The steps subsequent upon the decomposition of the 17β side chain were studied on epoxyandrostanes. As shown in Figs 2 and 4, on transformation of compounds IV and V, elimination of the oxido group was accompanied by 3-keto reduction. In the case of 4β,5β-epoxyandrostane-3,17-dione this early reduction was unexpectedly followed by repeated dehydrogenation, in accordance with the adverse function of 3α-hydroxysteroid-dehydrogenase. The newly formed 4β,5β-epoxyandrostane-3,17-dione (IV) was isolated after 24 hours, whereas 4β,5β-epoxy-3α-hydroxyandrostan-17-one (IX) after 4 hours of transformation. In the case of the latter compound, reduction of the 3-oxo group was confirmed by the infrared spectrum: there was a hydroxyl line and the oxo group was retained on the five membered ring D. The steric position of the hydroxyl group formed was confirmed in accordance with the finding of COLLINS *et al.*, that in the nuclear magnetic resonance spectrum of trans-4,5-epoxy-3-hydroxy compounds the proton at C_4 is marked by a singulette [22]. An approximately 15% reduction of the 3-oxo group was observed also with 4α,5α-epoxyandrostane-3,17-dione. This compound was isolated from transformations under anaerobic conditions [15].

Reduction of α-oxo-epoxysteroids has been reported by several authors [5, 6, 23], but no reference has been made to its relationship with the transformation of the oxido group. Our experimental results failed to disclose whether the elimination of the oxido group took place through a 4,5-epoxy-3-oxo or a 4,5-epoxy-3-hydroxy compound. On the one hand, reduction was incomplete (IV → IX; V → X), while on the other, there occurred a dehydrogenation of the 3α-hydroxyl group, as observed in cases IV → IX → IV, and VI → IX → IV (Figs 2 and 3). We shall discuss this problem in connection with our experiments under anaerobic conditions [15].

The rapid dehydrogenation of the 17β-hydroxyl group is a typical property of the *Mycobacterium phlei* strain used in the present study (VI → IX + + IV). In the experiments conducted under aerobic conditions, the 17-oxo compound was the sole demonstrable product [16, 17, 20].

No intermediate was found at the transformation of epoxysteroids to androst-4-ene-3,17-dione or to androsta-1,4-diene-3,17-dione. Formation of

androst-4-ene-3,17-dione was invariably observed and took place simultaneously with that of androsta-1,4-diene-3,17-dione. But experiments with *Mycobacterium phlei* have shown that formation of the Δ^1 double bond is preceded by that of Δ^4; hence it has been supposed that elimination of the oxido group results in androst-4-ene-3,17-dione which is transformed to androsta-1,4-diene-3,17-dione by dehydrogenation. The compounds $4\beta,5\beta$-epoxycholestan-3-one (I), $4\beta,5\beta$-epoxypregnane-3,20-dione (II) and $4\beta,5\beta$-epoxyandrostane-$3\alpha,17\beta$-diol (VI) invariably gave rise to a greater amount of androsta-1,4-diene-3,17-dione than $4\beta,5\beta$-epoxyandrostane-3,17-dione (IV) did. There is reason to suppose that in the case of compounds I and II, decomposition of the side chain increases the rate of the individual processes in some yet unidentified way.

The reported elimination of the oxido group finds little support in the literature. As already noted, in part of the examined cases the opening of the oxido group resulted in a dihydroxy compound [4, 5, 6]. Preliminary transformation experiments with some 4,5-dihydroxy derivatives of the substrates used did not result in the formation of Δ^4 derivative. WIX and ALBRECHT observed elimination of the oxido group during formation with *Fusarium caucasicum* of androsta-1,4-diene-3,17-dione, from $16\alpha,17\alpha$-epoxypregn-4-ene-3,20-dione. Details of this process were not examined, but in that case, too, there may have been a Δ^{16} intermediary compound, as the *Fusarium* strain transformed 3β-hydroxypregna-5,16-dien-20-one to androsta-1,4-diene-3,17-dione [8], *i.e.* to the same compound which had been obtained by transformation with our *Mycobacterium phlei* strain as well [17]. KADIS [3] explained the formation of 17α-hydroxypregn-4-ene-3,20-dione from 16α,-17α-epoxypregn-4-ene-3,20-dione in swine ovary tissue by a nucleophilic attack of the hydride ion originating from NADH at C_{16}. A similar process, which is novel in the case of microorganisms, cannot be excluded with our strain either (e.g. 3-hydroxy-4,5-oxido compound $\rightarrow$ 3,5-dihydroxy compound $\rightarrow$ Δ^{4-3}-oxo compound).

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NEW SELECTIVE MEDIUM FOR THE ISOLATION OF CLOSTRIDIUM PERFRINGENS

By

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Summary. Blood agar containing 800 μg D-cycloserine per ml has been found suitable for the selective cultivation of *Clostridium perfringens.* The medium inhibited the growth of Gram positive and, with the exception of *Serratia marcescens,* Gram negative organisms. In view of the resistance of *Serratia,* the medium was suitable for anaerobic cultivation by the Fortner technique. From human and animal faecal specimens and other materials *Cl. perfringens* was isolated rapidly in pure culture by selective inhibiting of the facultative anaerobic bacteria.

Clostridium perfringens is the most important member of the gas gangrene group of clostridia. It occurs frequently in wound infections and burns and is responsible for iatrogenic infections and food poisoning cases [1—5]. The overgrowth on solid media of miscellaneous bacteria present in most specimens make the isolation of *Cl. perfringens* difficult [1, 3—5]. Heating has long been used for the separation of sporulated clostridia from other organisms, but later the disadvantage of this method has been recognized and several selective media have been developed for isolation [6—21].

When studying the antibacterial spectrum of D-cycloserine we found that *Cl. perfringens* strains were more resistant to this antibiotic than facultative anaerobic bacteria. It was therefore of interest to examine the possible use of D-cycloserine for the selective isolation of *Cl. perfringens.*

Materials and methods

D-cycloserine sensitivity of Cl. perfringens. Ninety strains of *Cl. perfringens* were tested. Part of the strains were type cultures for types A, B, C, D and E, other strains were freshly isolated from soil, and human and animal faecal specimens.

Sensitivity determinations were carried out on the surface of blood agar (human blood, 5%; agar, 2%; Witte peptone, 1%; pH 7.4) plates. D-cycloserine bitartrate (Chinoin, Budapest) was dissolved in hot distilled water, then a series of plates containing the antibiotic at different concentrations was prepared. Before seeding the plates were dried at 37 °C for 1 hour. Then each plate was inoculated with a loopful of 24-hour thioglycollate broth cultures of the organisms. Incubation was performed in evacuated anaerobic jars for 24 hours at 37 °C.

Selective effect of D-cycloserine. Partly pure cultures, partly specimens containing a mixed bacterial flora were used. The bacterial cultures represented type strains and fresh isolates (Table I).

A total of 200 strains was examined on plates containing D-cycloserine at different concentrations. The inoculated media were placed in the anaerobic jar and read after incubation at 37 °C for 24 hours.

Preliminary studies on the inhibitory effect of D-cycloserine indicated that 800 μg/ml concentration ensured optimal selectivity. Faecal samples from 20 albino rats, 20 rabbits, 10 albino mice and 10 guinea pigs were suspended densely in saline and to 2 ml suspension 0.1 ml *Cl. perfringens* thioglycollate broth culture was added. Then one loopful of each specimen was streaked onto blood agar plates containing 800 μg per ml of D-cycloserine. Readings were made after 24 hours incubation at 37 °C. Faecal suspensions and *Cl. perfringens* cultures were seeded onto antibiotic-free plates as controls. Twenty throat swabs taken from young healthy individuals were also streaked onto blood agar plates with 800 μg D-cycloserine per ml. The plates were read after anaerobic cultivation for 24 hours.

Human faecal samples, which are known frequently to contain *Cl. perfringens*, were also examined. Twenty specimens were cultured in thioglycollate broth and after 16 hours incubation at 37 °C loopful amounts of the culture were transferred to selective and control plates. Readings were made after 24 hours anaerobic incubation at 37 °C. Suspected colonies of *Cl. perfringens* were identified by morphological properties and biochemical reactions (litmus milk, egg yolk medium and sugar fermentation).

Results

Cl. perfringens strains were highly resistant to D-cycloserine. Some strains grew even at 2000 μg/ml, but most of them were partly or completely inhibited at this concentration. At 1000 μg/ml the colony counts generally corresponded to those grown on the antibiotic-free control plates and only few strains were slightly inhibited. As all *Cl. perfringens* strains grew freely at 800 μg/ml, this concentration was adopted for our selective medium.

Aerobic Gram positive bacteria were generally more sensitive to cycloserine than clostridia. At 800 or 400 μg/ml none of them multiplied. At 200 μg/ml certain haemolytic streptococci and aerobic spore-forming bacteria grew fairly well. The most sensitive organisms were *C. diphtheriae* and *S. lutea* (Table I).

Gram negative bacteria were more resistant than Gram positive microorganisms. The examined *S. marcescens* strains grew at 2000 μg/ml. Other Gram negative bacteria were completely inhibited by 1000 or 800 μg/ml of cycloserine. *Proteus* and *Klebsiella* cultures were more resistant than other Gram negative bacteria. The examined *C. albicans* strains grew well at 2000 μg/ml concentration.

There was no growth on 800 μg/ml D-cycloserine plates seeded with throat swabs. On the antibiotic-free control plates an abundant growth of the throat flora was observed.

Cl. perfringens mixed into faecal specimens of various animals was recovered easily and in practically pure culture on selective plates: from 57 out of the 60 specimens only *Cl. perfringens* colonies developed, on the remaining 3 plates some minute lactobacillus colonies were also detected. *Cl. perfringens* colonies were typical in appearance and size and corresponded in number to colonies on the control plate inoculated with *Cl. perfringens* suspensions. Antibiotic-free blood agar plates seeded with faecal samples showed dense growth of miscellaneous faecal bacteria. Plates seeded with mouse or rat faeces were mainly characterized by a heavy swarming of *Pro-*

Table I

The in vitro sensitivity of various microorganisms to D-cycloserine
(Anaerobic cultivation on blood agar)

Organism	No. of strains	Minimum inhibitory concentration, μg/ml
Staphylococcus aureus	8	100
Staphylococcus albus	5	200
Staphylococcus citreus	1	100
Streptococcus pyogenes	10	400
Streptococcus viridans	10	200
Streptococcus faecalis	20	200
Diplococcus pneumoniae	2	200
Micrococcus tetragenus	1	100
Sarcina lutea	1	50
Corynebacterium diphtheriae	4	50
Corynebacterium hoffmanni	5	200
Listeria monocytogenes	5	200
Erysipelothrix rhusiopathiae	5	400
Mycobacterium phlei	1	200
Bacillus cereus	20	400
Bacillus subtilis	5	400
Escherichia coli	25	400
Klebsiella pneumoniae	12	800
Salmonella typhi	1	400
Salmonella paratyphi-B	1	400
Shigella flexneri	1	200
Proteus vulgaris	25	800
Pseudomonas aeruginosa	15	400
Pasteurella multocida	5	200
Brucella abortus	1	200
Neisseria pharyngis	5	200
Serratia marcescens	1	>2000
Clostridium perfringens	90	>1000
Candida albicans	5	>2000

teus. Other specimens yielded *E. coli*, *Klebsiella* and *Str. faecalis* and frequently viridans streptococci, lactobacilli and aerobic spore-forming bacilli. Rabbit faecal samples showed a similar picture but *Proteus* occurred rarely. From guinea pig faeces, mainly Gram positive bacteria were cultured on the control plates: staphylococci, enterococci, corynebacteria and aerobic spore-forming bacilli; Gram negative organisms were infrequent.

From human faecal samples we attempted to cultivate naturally occurring *Cl. perfringens*. The specimens were inoculated for enrichment into thioglycollate broth. Subsequent subcultures on cycloserine blood agar yielded *Cl. perfringens* colonies in 18 out of 20 samples; on the remaining two plates there was no bacterial growth. *Cl. perfringens* colonies on the selective medium were typical in every respect regarding both size and appearance. The colonies were generally surrounded by a characteristic haemolytic zone but some non-haemolytic colonies were also observed. On the control plates mainly *E. coli* and *Str. faecalis* colonies developed.

Discussion

In routine bacteriological examinations the isolation of *Cl. perfringens* is difficult as it calls for special technique and miscellaneous bacteria present in large numbers in various materials overgrow clostridial colonies. Owing to these difficulties, diagnostic isolation has not yet been introduced into practice as widely as it would be necessary.

Heating of materials has long been used for this purpose on the basis that the surviving spores grow in pure culture after the thermal destruction of non-sporulating bacteria. Unfortunately, heat treatment does not kill the areobic spore-forming bacteria often present in great numbers [3, 5, 12] and, on the other hand, *Cl. perfringens* does not produce spores readily. Aerobic sporulating bacteria were attempted to eliminate by making serial dilutions of the inoculum, but this technique is troublesome and time-consuming.

Recently, several selective media containing chloral hydrate, crystal violet, brilliant green, sodium azide, polymyxin-B, sulphadiazine or neomycin have been recommended. Unfortunately, the selectivity of these media is limited, as none of the above substances exerts a broad spectrum inhibitory effect. Chloral hydrate is weakly selective and is useful mainly for inhibiting the swarming of bacteria [5, 12]. Brilliant green inhibits primarily the growth of Gram positive organisms. To crystal violet, streptococci and some aerobic spore-forming bacilli as well as *Micrococcus tetragenus* and *Erysipelothrix rhusiopathiae* are fairly resistant [22, 23]. Sodium azide effectively inhibits Gram negative bacteria but it favours the proliferation of streptococci, micrococci, *E. rhusiopathiae* and *D. pneumoniae*. The applicability of this substance for the selective cultivation of *Cl. perfringens* seems doubtful in view of recent findings demonstrating azide sensitive clostridia [6—11, 22].

Polymyxin-B is active against Gram negative bacteria except *Proteus* strains. A combination of polymyxin-B and sulphadiazine did not improve selectivity considerably: on media containing these drugs not only *Proteus* but also resistant staphylococci, aerobic spore-forming bacilli and enterococci were observed [12—16, 24].

Of the above substances, neomycin shows the broadest spectrum of activity. This antibiotic is inhibitory to Gram negative bacteria and many Gram positive species. Primarily resistant organisms are only *Str. pyogenes*, *Str. viridans*, *D. pneumoniae* and *E. rhusiopathiae*, but many strains belonging to originally neomycin sensitive species have acquired resistance to the drug [17—21, 25].

D-cycloserine blood agar is very selective as it inhibits a wide variety of bacterial species and supports the growth of *Cl. perfringens*. The medium prepared with this antibiotic completely inhibited the growth of Gram positive bacteria including those resistant to other known selective agents. Of Gram negative organisms *Proteus* and other *Enterobacteriaceae* except *Serratia* were also effectively inhibited. As in diagnostic materials *Serratia* occurs infrequently, its resistance to D-cycloserine does not reduce the value of the method; on the contrary, it may be considered as a favourable property because it allows the application of Fortner's technique with the new medium.

The medium has been found effective with materials containing great numbers of miscellaneous bacteria, for instance throat swabs and faeces of animals contaminated artificially with *Cl. perfringens*. The results indicated that the new medium is suitable for direct plating and rapid isolation of *Cl. perfringens* from various materials. The examination of human faeces specimens suggests that the medium can be used for subculturing from thioglycollate broth, thus, for the examination of materials the low clostridial counts of which makes the use of enrichment media necessary.

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STUDIES ON SWINE SERUM IMMUNOGLOBULINS

II. RELATIONSHIP BETWEEN IMMUNOGLOBULINS AND ANTIBODIES

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Summary. The antibody nature of beta$_2$M and beta$_2$A globulin fractions, which had been previously considered immunoglobulins, was confirmed through direct reaction with the homologous antigen. By the same method, precipitins from certain swine sera readily precipitating bacterial antigens have been shown to be IgM and IgA immunoglobulins.

Also, during the development of immunity in swine, IgM macroglobulin was demonstrable earlier than IgA immunoglobulin. Experimental evidence is presented that in a certain stage of immunity two types of antibody to a single antigen may be present simultaneously, which are of identical specificity but of dissimilar immunoglobulin nature.

In a previous paper attempts to determine the protein components of swine serum by immunoelectrophoresis were reported in detail. Among the 28 components demonstrated on the basis of dissimilar immunological properties the 3 globulin types which corresponded to the 3 most important immunoglobulins — IgG, IgA and IgM — were readily distinguishable [7].

The present experiments were undertaken with the aim to obtain more information on the relationship between immunoglobulins and antibodies through the direct reaction of certain antibodies *in vitro.* For this purpose, antibodies readily precipitating the dissolved homologous antigens were looked for as in the gel diffusion test the precipitate can be fixed durably and also permits conclusions on the quantitative relationships. Also, it was of particular importance that in the gel diffusion system combined with electrophoresis the precipitin-containing globulin fractions were identifiable, thus a functional study of immunoglobulins was possible without previous separation.

Readily precipitating antibodies have been known to be present in swine erysipelas antisera. These precipitins react partly with the polysaccharide-hapten antigens responsible for the A and B types of *Erysipelothrix rhusiopathiae,* partly with their common antigen of protein nature. All these antigens are present in the dissolved state in the horse serum broth culture of *E. rhusiopathiae,* thus the 48-hour culture can be used directly as antigen in Ouchterlony's immunodiffusion test. Typing can be carried out also by bringing into reaction the pure culture of a single strain with A and B type sera as by the end of the reaction an additional precipitation line will appear on the side

of the specific serum. In contrast, sera from swine immunized with mixed cultures of different *E. rhusiopathiae* strains occasionally contain precipitins to all the three types of antigen and may accordingly give three precipitation lines with mixed *E. rhusiopathiae* cultures in the immunodiffusion test.

Precipitating swine erysipelas antisera of the above type were used with success in further studies of immunoglobulins. *E. coli* type sera from swine also proved favourable; they had been prepared with the aim to determine the different O and K antigens of *E. coli* strains by the agglutination test, but their good precipitating properties were soon detected.

In contrast with the above, so far no antiviral serum has been found to produce a visible precipitation reaction after the electrophoretic separation of serum proteins.

Materials and methods

Preparation of anti-swine-serum precipitating rabbit sera, as well as the immunoelectrophoretic method employed have been described in detail [7]. We applied SCHEIDEGGER's micromethod. Prior to the final drying of the plates the external agar strips along the canals were occasionally removed; this is the reason why they are not seen on some of the attached figures.

Immunodiffusion test. The agar-gel diffusion precipitation test was carried out according to OUCHTERLONY [4]. From purified powdered agar a 2% solution was prepared in pH 8.6 borate buffer, spread in 4.0 ml amounts over glass slides and allowed to solidify. In the agar layer, 15 cu.mm cylindrical wells were cut to hold the test antigens and antibodies. In accordance with the type of the test, either several wells were cut at 3—4 mm distance from the central well, or they were placed on both sides of the score passing along the slide's longitudinal axis. After placing the test substances into the wells, the slides were incubated in a moist chamber at room temperature for 40 hours. After the development of the precipitation lines, the free proteins were removed by dissolution in saline. Finally the slides were dried and the precipitation lines were stained with fuchsin according to the procedure described in connection with the immunoelectrophoresis method.

Bilateral examination of immune sera. Based on WILLIAMS and GRABAR's [11] working hypothesis, the relationship between immune serum precipitins and immunoglobulins was assessed by bilateral examination of the sera. According to common practice, electrophoretic separation of the swine serum was carried out in the midline of the agar plate. Then from one side precipitating rabbit serum, from the other side the homologous bacterial antigens were allowed to diffuse from the lateral grooves toward the swine serum fractions. The *E. rhusiopathiae* antigens were added to the system in the form of a 48-hour broth culture of 10^7—10^8 bacteria/ml density with 10% horse serum content. The *E. coli* antigen was added in the form of a suspension corresponding to 40 mg/1.0 ml wet bacterium concentration, as used also for the immunization of swine. In this experimental layout, the protein fractions of the swine serum interacted as antigens with the precipitating rabbit serum on the one side, while on the other side the precipitating swine antibodies entered into reaction with the bacterial antigens. The mirror-image like precipitation lines clearly indicated the serum fraction relationships of the swine serum antibodies. The bilateral examination of sera was also carried out in a system where bacterial antigens were placed in both lateral channels. This system allowed the identification of the reactions of the individual components of mixed antigens.

Immunization of swine. (a) A highly potent swine erysipelas antiserum was prepared according to HEGYELI and SURJÁN's method [3]. After hyperimmunization, the swine sera had an 80—100 I.U./ml protective value in the mouse test. Most of the hyperimmune sera readily precipitated the hydrochloric acid extract of *E. rhusiopathiae* grown in 10% horse serum containing broth [8] as well as the dissolved precipitinogens present in 48-hour cultures of this type.

(b) Agglutinating type sera suitable for strain identification were prepared in swine with the *E. coli* serotypes O111 B4; O55 B5; O26 B6; O86 B7; O127 B8; O112 B11; O128 B12; O119 B14; O125 B15; O126 B16 and O124 B17. In the pure culture of the *E. coli* strain of

checked serotype, antigen H was broken down by ethanol treatment. The bacteria were incubated in ethanol suspension twice for 2 hours at 37 °C, separated by centrifugation and resuspended in 5% dextrose solution to a concentration of 200 mg wet bacteria per ml. This suspension was distributed into 1 ml amounts and freeze-dried, thus each lyophile ampoule contained a dry antigen corresponding to 200 mg of wet bacteria, which comprised the O and B antigenic components of the given serotype.

Rising doses of the antigen calculated for kg body weight were inoculated into the pigs intravenously at 3—4 day intervals, giving antigen doses of 2.0, 4.0, 6.0, 8.0 and 10.0 mg/kg on the 1st, 5th, 8th, 12th and 15th day, respectively. The animals were exsanguinated on the 21st day, 6 days after the last antigen treatment.

Administration of caffeine simultaneously with the antigen had prevented the development of a shock-like reaction.

By this method a strictly type-specific antiserum, having an agglutinin titre of 1 : 2560—1 : 5120 in tube agglutination test was obtained. Antigens were kindly supplied by, and sera were checked in, the Department of Bacteriology, National Institute of Public Health, Budapest. These agglutinating sera were active also in the agar gel precipitation test. Examining them against a 40 mg/ml suspension of antigens used for the immunization of swine, one or more precipitation lines had formed. The precipitation reaction proved to be type specific, yet the precise nature of the relationship between the precipitinogens demonstrated and the O or B antigens remains to be clarified. Still, owing to their good precipitating ability, the sera were used with success in the present experiments.

From the blood of swine immunized according to the schemes described under (a) and (b), the serum was separated by natural clotting and used in the gel diffusion tests without preservation.

Treatment of sera with mercaptoethanol. According to BROWN *et al.* [1], inactivation of IgM was attempted by treatment with mercaptoethanol. The 0.2 *M* mercaptoethanol solution prepared in 0.04 *M* phosphate buffer, pH 7.6, was added in equal volume to the undiluted serum. The mixture was then allowed to stand for 2—3 hours at 20 °C and subsequently dialysed against 0.04 *M* phosphate buffer for 20 hours to remove the mercaptoethanol. The treated serum sample was then examined by immunoelectrophoresis. Comparison of the fractions present in the untreated serum and of their changes after treatment showed the sensitivity of the globulin fractions.

Results

1. Relation between precipitating antibodies to bacterial antigens and the immunoglobulins. (a) Bilateral examination of swine erysipelas antisera has shown that the antibodies which precipitate the dissolved antigens of *E. rhusiopathiae* correspond in most cases to the beta$_2$M globulin fraction of the swine serum, in other words they are IgM-type antibodies. Sera from most examined swine gave two precipitation lines in the IgM region. In part of the cases the two lines run closely parallel, in part they deviated slightly toward the direction of the electrophoretic migration and intersected. Often a shorter, more curved line was seen along the concave side of the longer, less curved line. The shorter line consistently appeared regardless of the antigen with each serum sample from one and the same donor, whereas the external longer line appeared or disappeared depending on the type of the strain. This permitted the conclusion that the reaction of the protein antigen, which is independent of the type, separates from that of the type specific hapten-like antigen and two precipitation lines with different radii are resulting.

Summarizing the results obtained with the individual swine sera, it appears that the precipitins to the different antigens of *E. rhusiopathiae* bacteria enter into reaction with the homologous antigen separately and the

precipitation lines formed appear either independently or coincide partially or wholly, depending on the concentration relations of the antigen-antibody pairs.

In addition to the IgM type reactions, swine erysipelas antisera occasionally showed in the gamma globulin region a faint precipitation line which mostly faded away. Such lines formed as a rule very closely to the groove containing the antigen, but on 2—4-fold dilution of the serum they withdrew slightly. Their appearance seems to suggest that the examined sera contained also IgG type antibodies to *E. rhusiopathiae*, but the applied method did not allow their closer study.

(b) A particularly favourable model for the study of immunoglobulins was presented by swine type sera to O and B antigens of *E. coli*. Demonstration

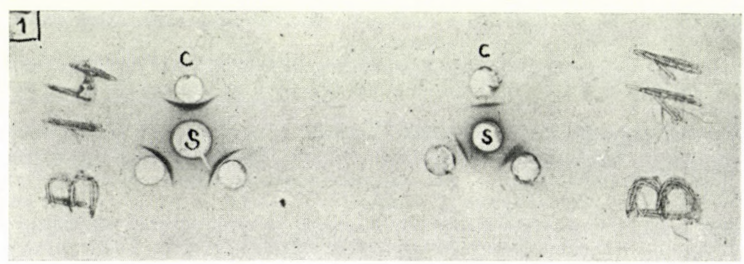

Fig. 1. Radial immunodiffusion test of *E. coli* O119 B14 and *E. coli* O112 B11 antigen solutions with homologous swine immune sera. Explanations: + = anode in electrophoresis; S = swine serum; L = rabbit serum; C = *E. coli* bacterial antigen; A = reaction of IgA type precipitins; M = reaction of IgM type precipitins; 1—64 = reciprocal values of swine serum dilutions

of the precipitins of these type sera was attempted first by double immunodiffusion. If the antigen suspension prepared from the appropriate type of *E. coli* was placed in 3 wells around the central well which contained the swine serum, three kinds of precipitation line appeared in several characteristic patterns. Often a single, distinct line was seen immediately adjacent to the antigen well (Fig. 1, B14); occasionally an additional faint line was seen behind, at about the middle of the agar area. Other sera produced only the latter straight line (Fig. 1, B11), or inside it another line directed toward the well containing serum.

To decide to what an extent the positions of the precipitation lines were dependent on the serum antibody contents, the precipitating capacity of swine sera was examined also by the serial dilution method. Twofold serial dilutions of the serum were placed in succession in the row of wells along the longitudinal groove containing the antigen. In this system, the shift of the individual precipitation lines toward the serum-containing wells connected with the decrease of antibody concentration was clearly apparent (Figs 2, 3). Nevertheless, the three types of precipitation line localization still persisted. The line

located nearest to the serum-containing well had such a pronounced curvature that it did not meet the corresponding line of the next dilution step even in the low dilution range (see M on Figs 2, 3, 4). In the high dilution range it appeared more and more as a crescent closely adjacent to the serum well until it disappeared completely.

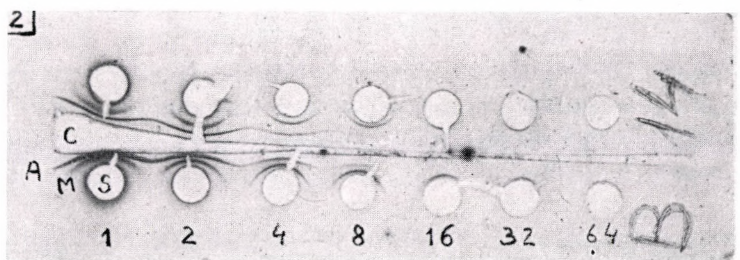

Fig. 2. Immunodiffusion test of *E. coli* O119 B14 antigen solution with serially diluted homologous swine immune serum. For explanations see Fig. 1

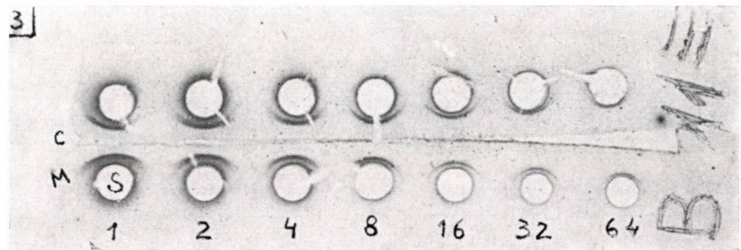

Fig. 3. Immunodiffusion test of *E. coli* O112 B11 antigen solution with serially diluted homologous swine immune serum. For explanations see Fig. 1

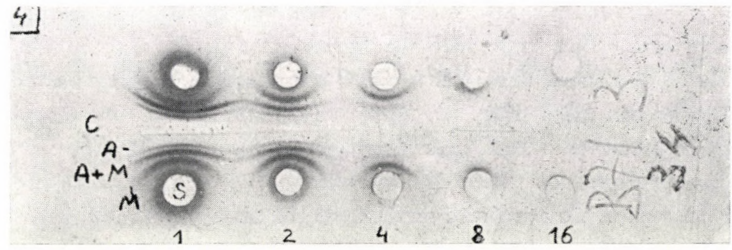

Fig. 4. Immunodiffusion test of *E. coli* O86 B7 antigen solution with serially diluted homologous swine immune serum. For explanations see Fig. 1

Another precipitation line which was frequently seen formed close to the antigen groove and hardly retreated from it even in the high dilution range. The corresponding lines of the adjacent dilution steps merged to an undulating line which was typical of the identity reaction (see A in Figs 2 and 4).

With some serum samples a third type of precipitation line was also seen which formed roughly in the midline between serum well and antigen groove

(Fig. 4, A + M). It occurred either together with both lines of the other type or only with one of them. Further studies of this phenomenon suggested that the formation of the three different types of precipitation line was related to class differences of immunoglobulins.

The reactions obtained with serial dilution and the disappearance of certain types of precipitation line at different degrees of dilution indicated the variation of the relative amount of precipitins in the individual sera. Under favourable circumstances, however, the simultaneous presence of at least three antigen-antibody systems was demonstrable (Fig. 4).

To obtain more information on the demonstrated precipitins, the immune sera were examined by the bilateral method. From the positions of the pre-

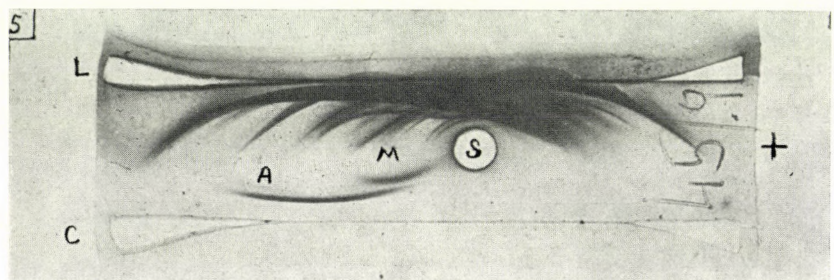

Fig. 5. Bilateral examination of swine *E. coli* antiserum, developed with homologous antigen (bottom) and anti-swine rabbit serum (top). For explanations see Fig. 1

cipitation lines it was concluded to the presence of IgA and IgM type antibodies (Fig. 5) which showed an individual variation of the precipitation pattern. Nearly all serum samples displayed one or two IgM type lines (Fig. 6). Often an additional IgA type line was present (Fig. 7). Exceptionally there was a fourth line, also of the IgA type. Such a rich precipitation picture was shown by the serum B7/3 (Fig. 8). Since with this serum the merging of the internal IgA and external IgM line signified an identity reaction, the pattern confirmed the presence of three different types of antigen, consistently with that shown on Fig. 4. Toward one antigen the serum contained only IgA type, while toward the other only IgM type precipitin. But precipitins to the third antigen were of both IgA and IgM type.

With sera showing a single IgA line and two IgM type lines, the former usually gave an identity reaction with the external IgM line (Fig. 9). Thus the general conclusion may be drawn that in a given stage of the immune response, in swine sera two types of antibody to a single antigen may be present simultaneously, whose immune specificity is identical, while their molecular structures are dissimilar, being derived from different immunoglobulin classes.

The fact that the demonstrated precipitins belonged to two classes of immunoglobulin also throws a light on the circumstance that the typical

6

7

8

Figs 6, 7 and *8*. Immunoelectrophoretic examination of three swine *E. coli* antisera, each developed with the homologous antigen. For explanations see Fig. 1

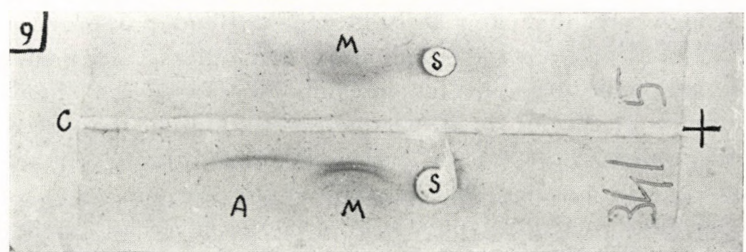

Fig. 9. Early (top) and late (bottom) immunoelectrophoretic patterns of a swine *E. coli* antiserum developed with the homologous antigen. For explanations see Fig. 1

position of certain lines in the double immunoprecipitation test is determined by the different diffusion constants of the different precipitin types. Accordingly, the reaction appearing close to the serum wells is produced by the slowly diffusing IgM precipitins, whereas that close to the antigen well by the more rapidly diffusing IgA precipitins.

Finally, the reaction appearing in about the midline between the antigen and serum wells is that produced by a single antigen with two types of immunoglobulin (Fig. 4).

2. Sequence of the appearance of the different immunoglobulins during the development of immune response. A shift was observed in the temporal appearance of the above described two different immunoglobulin types of antibody. In sera from swine immunized on 5 occasions for *E. coli* type serum production exclusively IgM type antibodies were demonstrable by immunoelectrophoresis on the 11th day after the first immunization. After further 7 or 9 days, IgA type precipitins appeared with a distinct reaction in nearly all swine sera. Simultaneously the reactions of IgM precipitins became more pronounced, indicating a further increase in their amounts (see Fig. 9: top: early blood sample; bottom: late blood sample).

The macroglobulin nature of the early IgM type antibodies was supported by the experiment confirming their sensitivity to mercaptoethanol. As described in the foregoing, serum samples were allowed to react at room temperature with equal volumes of 0.2 *M* mercaptoethanol. Incubation for two and a half hours was sufficient for the disappearance of the reaction attributed to IgM antibodies as demonstrated by the absence of the respective precipitation line in the immunoelectrophoretogram. In the meantime, the precipitation reaction attributed to IgA antibodies had become less distinct, but was still recognizable.

Discussion

The demonstration of precipitating antibodies to bacterial antigens in the above systems offers direct evidence of the presence of antibodies in the $beta_2$ globulin group. Experiments with *E. coli* type sera disclosed that in the early stage of immunity $beta_2$ M macroglobulin, *viz.* IgM globulin is formed at a high rate, thenl ater also IgA type antibodies appear to the same antigen. Thus in a certain stage of immunity antibodies of identical specificity but originating from different immunoglobulin classes are simultaneously present in the swine serum. On electrophoretic separation these two immunoglobulins react with the common antigen by each forming an independent precipitation line in a region characteristic of the immunoglobulin type, but at the site of their encounter the two lines merge in a way characteristic of the identity reaction. OUDIN [5] found that in the radial immunodiffusion test the reactions of the different antibodies to one single antigen appeared

in the form of a single line when a mixture of antibodies to the different determinant groups of the antigen had been brought into reaction. Our results suggest that OUDIN's finding applies also to the reaction of different Ig type antibodies to the same antigen. In such cases the single line develops in the midline between the wells containing the serum and the antigen, unlike the reaction of antibodies of "pure" Ig type, which enter into reaction with the antigen in regions determined by their diffusion rates. Accordingly, the IgM macroglobulin covers only a slight distance beyond the serum containing well, whereas the IgA type precipitin forms the precipitation line close to the antigen containing well.

Though the presence of antibodies other than the above was hardly or not at all demonstrable in the precipitation test the results of several earlier experiments suggest that the sequence of production of the different immunoglobulins applies to a broader range of antibodies. Thus *e.g.* the antibodies present in hyperimmune anti-swine fever or anti-Aujeszky sera usually precipitate the suspension of the homologous virus in the double immunodiffusion test, while precipitation lines fail to appear on immunoelectrophoresis, where the antibody concentration per unit area is low. However, the immunoelectrophoretograms of the same antiviral sera developed with rabbit serum usually show an elongation and thickening, *i.e.* a quantitative increase, of the IgM line in the early stage of immunity already. Earlier studies with free electrophoresis [6] already suggested that at the first manifestation of the serum's protective action exclusively the beta-globulin had increased. It was then inferred that the early antibodies produced in large amounts under the influence of hyperimmunization correspond to the late antibodies exclusively in biological effect, but differ from them in their physical and chemical properties. The present knowledge on 19S and 7S immunoglobulins [2, 9, 10] as well as the experiments described in this paper offer an indirect proof and a plausible explanation of that earlier hypothesis.

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STUDIES ON THE PERITONEAL EXUDATE OF ANIMALS EXPERIMENTALLY INFECTED WITH TOXOPLASMA GONDII

II. SUSCEPTIBILITY OF RATS AND CHICKENS

By

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Summary. Gradually increasing doses of the supernatant of mouse toxoplasmic peritoneal exudate were injected intravenously in equal groups of chickens. The material induced respiratory and nervous disturbances and in proper doses, death. Rats also proved susceptible to the material if injected by the venous route. Intraperitoneal injection did not cause discomfort either in chickens or in rats. Freezing (—20 °C) and thawing reduced but did not abolish the toxic effect to rats. The significance of the findings is discussed.

The supernatant of the peritoneal exudate of animals infected with *T. gondii* induced respiratory and nervous disturbances and killed mice when injected intravenously [1, 3—9]. Animals producing a potent exudate include mice, guinea pigs, young rats treated with cortisone, and rabbits [6]. However, no attempt has been made to test the susceptible animal range. The aim of the present study was to test the susceptibility of rats and chickens.

Materials and methods

Mice. Male and female Swiss white mice 20—25 g in weight were used for the production of peritoneal exudate supernatant (SPET) [1] and for its assay [6] before testing it on other animals.

Rats. Female albino rats were tested for susceptibility to SPET by injection into the lateral caudal vein. A total of 40 young rats, 55—90 g in weight were used. Ten rats were injected intravenously each with 0.01 ml per g body weight of SPET. Another 10 rats were injected intravenously each with 0.01 ml per g body weight of twice frozen and thawed SPET. This material did not kill any of the rats and therefore it was injected intravenously to each of 10 rats in a dose of 0.012 ml per g body weight. Ten rats each were injected intraperitoneally with 2 ml fresh SPET and observed for 24 hours.

Chickens. Young chickens of 42 to 55 g in weight, were tested for susceptibility to SPET by injection in the vein of the wing. Gradually increasing doses between 0.002 and 0.006 ml per g body weight were injected in groups of 5 chickens and in each group the number of deaths was recorded. Ten chickens were injected intraperitoneally each with 1 ml fresh SPET and observed for 24 hours.

Table I

Effects of 0.01 ml/g b.w. of fresh SPET on rats

Rat No.	Weight, g	Symptoms	Time of death
1	60	apnoea, circus movements fell on its side, gasping, urination, exophthalmus, death	45″
2	60	apnoea, wobbling, circus movement, gasping, urination, death	6′
3	55	inert, apnoea, hing quarter paralysis, circus movement, gasping, sanguineous froth out of nose and mouth, death	7′
4	55	inert, apnoea, circus movement, hind quarter paralysis, recovery	
5	70	wobbling, inert, slow extension of hind limbs, irregular gasping, recovery	
6	72	wobbling, apnoea, gasping, death	1′
7	85	prone, apnoea, hind quarter paralysis, recovery	
8	90	wobbling, weak hind limbs, recovery	
9	60	apnoea, irregular rapid respiration, weak hind limbs, recovery	
10	60	apnoea, irregular rapid respiration, recovery	

Table II

Effects of 0.01 ml/g b.w. of twice frozen (—20 °C) and thawed SPET on rats

Rat No.	Weight, g	Symptoms
1	70	apnoea, irregular respiration, recovery
2	70	apnoea, irregular respiration, weak hind limbs, recovery
3	65	irregular respiration, weak hind limbs, recovery
4	60	tachypnoea, recovery
5	90	tachypnoea, recovery
6	72	irregular respiration, weak hind limbs, recovery
7	80	tachypnoea, flexion of fore limbs, extension of hind limbs, recovery
8	60	irregular respiration, recovery
9	55	apnoea, weak hind limbs, recovery
10	75	tachypnoea, recovery

Table III

Effects of 0.012 ml/g. b.w. of twice frozen (—20 °C) and thawed SPET on rats

Rat No.	Weight, g	Symptoms	Time of death (minutes)
1	75	apnoea, irregular respiration, recovery	
2	70	apnoea, irregular respiration, recovery	
3	80	laboured respiration, hind limb paresis, recovery	
4	60	apnoea, irregular respiration, gasping, death	11
5	72	tachypnoea, flexion of toes of fore limbs, recovery	
6	80	tachypnoea, hind limb paresis, recovery	
7	95	wobbling, tachypnoea, recovery	
8	95	apnoea, tachypnoea, recovery	
9	85	tachypnoea, recovery	
10	90	tachypnoea, recovery	

Results

Both rats and chickens proved susceptible to SPET.

Rats. Fresh SPET in a dose of 0.01 ml per g body weight killed 4 out of 10 young rats (Table I). The same dose of frozen-thawed SPET killed none of 10 rats (Table II), while a dose of 0.012 ml per g body weight killed 1 out of 10 rats (Table III). Death occurred 1—7 minutes after injection of fresh SPET and 11 minutes after injection of frozen-thawed SPET. Intraperitoneal injection of SPET caused no discomfort.

Manifestations in rats following the intravenous injection of SPET. The symptoms in rats were less stormy than those [1] in the mouse. Following the injection of a lethal dose, the rat became apnoeic, ran around on the table, sometimes around a vertical axis (circus movement), then fell on its side or remained inert. Then it started gasping which gradually faded and the animal died. Sometimes, there was urination and exophthalmos. One rat died with sanguineous frothing from nose and mouth.

Following the injection of a sublethal dose the rat developed apnoea or tachypnoea, remained motionless for a few seconds, with the fore limbs flexed and the hind limbs extended backwards, irregular gasping started and the rat wobbled, dragging its hind limbs; then it recovered, but tachypnoea persisted for some time.

Chickens (Table IV). Fresh SPET killed chickens in a dose of 0.006 ml per g body weight and 2 out of 5 chickens in a dose of 0.004 ml per g body weight. Death occurred in 40 seconds to 2 minutes. Intraperitoneal injection of SPET caused no discomfort.

Table IV

Effects of fresh SPET on chicken

Chicken wt	Dose/g b.w.	Symptoms	Time of death (seconds)
45		+++	45
45		+++	190*
46	0.006	+++	40
46		+++	30
46		+++	110*
48		+	—
46		+	—
46	0.004	++	95*
55		++	110*
43		+	—
42		—	—
43		—	—
46	0.002	—	—
46		—	—
42		—	—

wt = weight; g b. w. = gram body weight; +++ = convulsions, apnoea and death; ++ = weakness, dyspnoea, and death; + = weakness, dyspnoea, no death; — = no apparent discomfort; * = two-stage syndrome (see text)

Manifestations in chickens following the intravenous injection of SPET. In chickens the lethal dose immediately elicited convulsions with apnoea. Then the convulsions decreased in severity, the chicken remained on its side with convulsing wings and legs, then the bird defecated and after some gasping it died. Four chickens showed a two-stage syndrome, with a first stage with convulsions and apnoea, then they opened their eyes, regained a regular, though laboured respiration and remained on their side for a few seconds. Then a second stage of convulsions appeared which ended in death.

Following the injection of a sublethal dose, the chicken closed its eyes, leaned on its side and rested its head on the table. Respiration was slow and laboured. No convulsions but sometimes defecation occurred. Finally the bird suddenly opened its eyes and stood up on its legs, it recovered within 2 minutes though prostration persisted for a little time.

Discussion

Rats and chickens were found susceptible to the intravenous injection of SPET which induced respiratory and nervous disturbances and in higher doses rapid death. Intraperitoneal injections did not cause discomfort either

in these species or in the mouse [3]. The active material, which was considered toxic and termed "toxotoxin" [3], has been recovered in the globulin fraction of the exudate [6]. Hyaluronic acid was found important in its structure [7]. Its toxic nature is, however, not universally accepted and the exact mechanism of action is far from clear. The sequence of events after intravenous injection of SPET is too rapid to be of a true toxic nature. VARELA *et al.* [9] compared it to the similar embolic effect of egg white in mice and FULTON [7] to the syndrome in chickens elicited by the intravenous injection of Rous sarcoma extract and concluded that SPET caused circulatory disturbances. WEINMAN [5] and FULTON [7] reported, however, that heparin does not protect against its lethal effect. FULTON [7] stated that histamine, LSD, 5HT, bradykinin and bradykinin-forming enzymes have no role in its activity and found that it is not haemolytic. WEINMAN [5] found that SPET does not agglutinate chicken red cells beyond 1/10. We found that half of the mice receiving single sublethal doses passed red urine with red cell casts. Splenectomy done before the injection of SPET failed to inhibit this phenomenon [2].

The syndrome which follows the intravenous injection of SPET is different from that caused by toxoplasmosis and it is doubtful if it plays any role in its pathogenesis. It rather resembles anaphylactic, peptone or histamine shock in every animal studied, thus in the mouse [1, 8], the rat [16, 17, 18, 19, 20] and the chicken [12, 13, 14]. One important difference from anaphylactic shock is, however, that SPET needs no sensitization.

Freezing and thawing reduced but did not abolish toxicity or lethality of SPET in the rat; this corresponds to previous findings in mice [1].

We do not agree with the view [15] that the fact that more than one animal species is susceptible to SPET should speak for or against its toxic nature. Research should, however, be extended to large susceptible animals in order to solve the enigma of SPET.

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COLICIN SENSITIVITY AND COLICINOGENITY OF TYPE O124 : K72(B17) E. COLI STRAINS

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Summary. A total 505 *E. coli* O124 strains isolated in different parts of Hungary were examined for colicin sensitivity and colicinogenity. Of the strains, 54.2% proved to be colicinogenic and 96% sensitive to two or more colicins of which 31 types were identified. Colicin typing appears to be helpful for the epidemiological analysis of *E. coli* O124 strains.

Among the so-called enteropathogenic *E. coli* strains, strain O124 : K72(B17) (further referred to as *E. coli* O124) may cause enteritis not only in infants, but also in adults. The clinical course is often simulating dysentery [2, 9, 10, 12, 16]. Like shigellae, the majority of *E. coli* O124 strains cause keratoconjunctivitis in the guinea-pig [18] and is antigenically related to *Sh. dysenteriae* 3 [4]. Based on these properties, some investigators regard *E. coli* O124 as transitory types between the species Shigella and Escherichia [1, 13, 19]. In Hungary, *E. coli* O124 strains were classified on the basis of their lysogenic properties [3, 11].

The production of colicin by bacteria of their sensitivity to known colicins has increasingly been employed for differentiation. In the present paper studies on the colicin status of *E. coli* O124 strains are reported.

Materials and methods

The *E. coli* O124 strains examined were isolated from minor outbreaks, sporadic diseases, and symptomless carriers. The majority was isolated from adults, the minority from nursery and schoolchildren. Of the 505 strains 264 were isolated in county Nógrád, the remaining 241 in other parts of Hungary.

The reference colicinogenic strains used for typing are listed in Table I.

The colicin sensitivity of the strains was determined by Fredericq's double layer agar method [6] slightly modified by us. The reference colicinogenic strains were inoculated by stabbing into an agar medium containing 7% bovine blood. For this purpose we prepared a replicator tool bearing 9 thin steel needles; this allowed the simultaneous inoculation of 9 colicinogenic strains in uniform distribution over the Petri dish. The macrocolonies which developed after incubation for 24 hours at 37 °C were sterilized for 1 hour in chloroform vapour, then the chloroform was removed by aeration. The 4-hour broth culture of the *E. coli* O124 strain to be tested was diluted 1 : 5 and overlayed by 2.5 ml of 0.7% soft agar cooled to 50 °C. Results were read after incubation for 18 hours at 37 °C.

Colicinogenity was determined with the *E. coli* K-12 Row strain.

Table I

Colicinogenic reference strains used for typing

Strains		Colicin produced	Origin
Designation	Name		
CA7	*E. coli*	V	P. Fredericq, Liège
CA18	*E. coli*	B	P. Fredericq, Liège
CA23	*E. coli*	D	P. Fredericq, Liège
CA31	*E. freundii*	A	P. Fredericq, Liège
CA38	*E. coli*	E+I	P. Fredericq, Liège
CA42	*E. coli*	F	P. Fredericq, Liège
CA46	*E. coli*	G	P. Fredericq, Liège
CA53	*E. coli*	I	P. Fredericq, Liège
CA57	Paracoli	C	P. Fredericq, Liège
CA58	*E. coli*	H	P. Fredericq, Liège
CA62	Paracoli	J+I	P. Fredericq, Liège
K235	*E. coli*	K+?	P. Fredericq, Liège
P1	*Sh. boydii*	S_1	P. Fredericq, Liège
P9	*Sh. sonnei*	S_3+I	P. Fredericq, Liège
P14	*Sh. dispar*	S_5	P. Fredericq, Liège
P15	*Sh. dispar*	S_4	P. Fredericq, Liège
K30	*E. coli*	V+E_1	L. Alföldi, Szeged
ML	*E. coli*	E_1	L. Alföldi, Szeged

Table II

Sensitivityof 505 E. coli O1 24 strains to the 16 Fredericq-type colicins

Colicin	Sensitive strains		Colicin	Sensitive strains	
	Number	per cent		Number	per cent
V	478	94.7	C	467	92.4
B	17	3.3	H	421	83.3
D	2	0.3	J+I	484	95.8
A	2	0.3	K+?	33	6.5
E+I	471	93.2	S_1	3	0.6
F	480	95.0	S_3+I	480	95.0
G	375	74.2	S_5	8	1.6
I	373	73.8	S_4	2	0.3

Results

Colicin sensitivity and colicinogenity were determined for 505 *E. coli* O124 strains. The results obtained with the 16 Fredericq-type colicins are shown in Tables II and III.

The most effective colicins were J+I (95.8%), S_3+I (95.0%), F (95.0%), V (94.7% and E+I (93.2%). A lesser number of *E. coli* O124 strains was sensitive to D, A, S_4, S_1, S_5 and B colicins. Average sensitivity of the strains was to 8 colicins, and 54.3% of them were sensitive to 9 colicins. Twenty strains (4%) were not sensitive to any of the colicins tested. Only one strain was sensitive to all colicins tested.

Table III

Distribution of the examined E. coli O124 strains according to colicin sensitivity

Number of effective colicin	Number of sensitive strains	Number of effective colicin	Number of sensitive strains
1	0	9	274
2	1	10	21
3	3	11	5
4	0	12	0
5	12	13	2
6	10	14	0
7	56	15	0
8	100	16	1

Of the 505 strains, 267 (52.4%) were colicinogenic. The strains were classified into 31 types on the basis of colicin sensitivity and colicinogenity (Table IV). The colicinogens CA-7 and K-30 had nearly identical effects. The pure E_1 colicin produced by the strain ML *E. coli* acted except for types 9 and 10 (10 strains) only on those colicin sensitive strains which were non-colicinogenic. The colicin-resistant strains could also be divided into two groups according to their producing or not producing colicin.

Discussion

As colicinogeny is a stable hereditary property of bacteria, determination of the colicinogen type would be a more reliable method than colicin typing. Since, however, the typing of the produced colicin(s) is difficult [7] it is not suitable for routine application. In addition, only 50–70% of the strains are colicinogenic. Thus, determination of the colicin type seems to be recommendable. When freshly isolated strains are used under rigorously observed ex-

Table IV

Classification of 505 E. coli O124 strains examined for colicin sensitivity and colicinogenity

Colicin type	V	B	D	A	E+I	F	G	I	C	H	J+I	K+?	S_1	S_3+I	S_5	S_4	E_1+V	E_1	Colicinogenity	Number of strains
1	+	—	—	—	+	+	—	—	+	+	+	—	—	+	—	—	+	—	+	19
2	+	—	—	—	+	+	+	+	+	+	+	—	—	+	—	—	+	—	+	104
3	+	—	—	—	+	+	+	—	+	—	+	—	—	+	—	—	+	—	+	25
4	+	—	—	—	+	+	—	+	+	+	+	—	—	+	—	—	+	—	+	42
5	+	—	—	—	+	+	+	—	+	—	+	+	—	+	—	—	+	—	+	4
6	+	—	—	—	+	+	+	—	+	+	+	—	—	+	—	—	+	—	+	13
7	+	—	—	—	+	+	+	+	+	—	+	—	—	+	—	—	+	—	+	17
8	+	+	—	—	+	+	+	—	+	+	+	—	—	+	—	—	+	—	+	8
9	+	—	—	—	+	+	+	+	+	+	+	—	—	+	—	—	+	+	+	5
10	+	—	—	—	+	+	—	+	+	—	+	—	—	—	—	—	+	—	—	5
11	+	—	—	—	+	+	+	+	+	+	+	—	—	+	—	—	+	+	—	153
12	+	—	—	—	+	+	+	+	+	+	+	+	—	+	—	—	+	+	—	21
13	+	—	—	—	+	+	+	+	+	+	+	+	—	+	+	—	+	+	—	5
14	+	—	—	—	+	+	—	+	+	+	+	—	—	+	—	—	+	+	—	12
15	+	—	—	—	+	+	—	—	+	+	+	—	—	+	—	—	+	+	—	9
16	+	—	—	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+	—	1
17	+	—	+	—	+	+	+	+	+	+	+	+	+	+	+	—	+	+	—	1
18	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	—	1
19	+	+	—	+	—	+	—	—	—	+	+	—	—	+	—	—	+	+	—	1
20	+	+	—	—	+	+	+	—	+	+	+	—	—	+	—	—	+	+	—	4
21	+	—	—	—	+	+	+	—	+	+	+	—	—	+	—	—	+	+	—	12
22	—	—	—	—	—	—	—	—	—	+	+	—	—	+	—	—	—	—	+	3
23	+	—	—	—	+	+	—	—	—	—	+	—	—	+	—	—	+	—	+	9
24	—	—	—	—	—	+	—	+	+	—	+	—	—	+	—	—	+	—	+	3
25	+	+	—	—	—	+	—	—	—	+	+	—	—	+	—	—	+	—	+	3
26	+	—	—	—	+	+	—	—	+	+	—	—	—	+	—	—	+	—	+	1
27	+	—	—	—	—	—	+	+	—	+	+	—	—	+	—	—	+	—	+	1
28	—	—	—	—	—	—	—	—	—	—	+	—	—	+	—	—	—	—	+	1
29	+	—	—	—	—	+	—	+	+	+	+	—	—	+	—	—	+	—	+	2
30	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	7
31	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	13

\+ = sensitive to colicin
— = resistant to colicin

perimental conditions, Escherichia show a lesser heterogeneity to colicins than Shigellae [17]. Ninety-six per cent of the *E. coli* O124 strains were sensitive to two or more colicins. Our results are in good accordance with the observations on pathogenic *E. coli* serotypes of other authors [5, 8, 14, 15, 20]. Determination of colicin sensitivity makes it possible to differentiate between *E. coli* O124 strains; this is important in the epidemiological context, particularly if the strains are neither lysogenic, nor colicinogenic.

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INDEX

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Rédai, I., Réthy, A., Rozgonyi, F.: Fatty Acids in *Staphylococcus aureus* and *Escherichia coli* Cultured *in vivo* 219

Rosztóczy, I.: Effect of Emetine on the Multiplication of Pseudorabies and Semliki Forest Viruses and on the Interferon Production by Cultured Cells 227

Raskó, I., Kerekes, E., Alföldi, L.: Properties of L-Serine Deaminase from *Salmonella typhimurium* and *Bacillus cereus* 237

Koch, A., György, E.: Heart Glycosides in Poliovirus Host Cell Interaction. III. Chemical Structure and Activity 245

Büki, K. G., Ambrus, G., Szabó, A.: Microbiological Decomposition of 17α-methyl-17β-hydroxy Steroids with Androstane Nucleus 253

Tömörkény, E., Albrecht, K., Ila, L.: Transformation of 4,5-Epoxysteroids with *Mycobacterium phlei*. I. Transformation under Aerobic Conditions 261

Füzi, M., Csukás, Zs.: New Selective Medium for the Isolation of *Clostridium perfringens* 273

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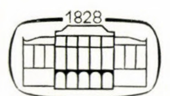

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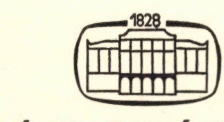

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ANTIGENIC RELATIONSHIP BETWEEN SHIGELLA SONNEI PHASE II AND ENTEROBACTER CLOACAE

By

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(Received March 27, 1969)

Summary. Flagellated *Enterobacter cloacae* strain 6302/RB contained an L-type envelope antigen and an O antigen showing an "ab–ac" relationship to *Shigella sonnei* phase II. Diagnostic problems connected with the finding have been discussed.

In previous papers we have pointed out that the application of routine serological examinations (slide agglutination, microagglutination and immunofluorescent tracing) without other tests may cause diagnostic errors [1—4].

Findings described in this paper have confirmed this opinion and have indicated that even the usual orientation biochemical tests may be insufficient to establish a correct bacteriological diagnosis.

Materials and methods

Isolation of E. cloacae. In May, 1967, a female patient was admitted to the infectious disease department of the county hospital with severe dysenteric symptoms. Faecal cultures on desoxycholate citrate agar were negative, but on Endo agar lactose negative colonies developed. The triple (sugar-urea-iron) differentiating medium subculture was characteristic of shigellae. The culture agglutinated in *Sh. sonnei* I—II serum. A preliminary report including antibiotic sensitivity was given. As despite aimed antibiotic therapy the patient's condition aggravated and as the clinical picture was atypical, the isolated strain was studied further. The culture failed to cause keratoconjunctivitis on the guinea pig's eye and was atypical in biochemical behaviour. Thus a final report of "Shigella negative" was given, then further laboratory examinations were performed which revealed that the patient suffered from amoebic dysentery.

Biochemical and serological examinations were performed by the usual methods [5—13]. Agglutination was carried out in 0.2% NaCl.

Type strains were maintained in the collection of our institute. Strain *Plesiomonas shigelloides* was kindly supplied by Dr. F. W. Winton (University Medical School, Edinburgh).

Results

Biochemical properties of strain 6302/RB are presented in Table I.

The data indicate that the culture belongs to *E. cloacae*: negative lysine decarboxylase, positive ornithine decarboxylase and positive arginine dihydrolase reaction: late (72 hour) gelatin liquefaction and negative glycerol reaction.

Table I

Biochemical properties of strain 6302/RB and E. cloacae

	1	2		1	2
Motility, semisolid agar, 37°C	+	+	Glucose acid	+	+
			gas	+	+
Catalase	+	+	Adonitol	+	
Oxidase	—	—	Arabinose	+	+
NO_3 reduction	+	+	Dulcitol	—	—
KCN	+	+	Glycerol	—	—
H_2S from thiosulphate	—	—	Inositol	—	—
Urease	—	—	Lactose	(+)	+
Indole	—	—	ONPG	+	+
Lysine decarboxylase	—	—	Mannitol	+	+
Arginine dihydrolase	+	+	Raffinose	+	+
Ornithine decarboxylase	+	+	Rhamnose	+	+
Phenylalanine deaminase	—	—	Sucrose	+	+
Gelatinase	(+)	(+)	Salicin	+	+
Lipase	—	—	Sorbitol	+	+
Citrate utilization			Methyl red 37°C	—	—
(Simmon's medium)	+	+	22°C	—	—
Malonate test	+	(+)	Voges–Proskauer 37°C	+	+
Mucate test	+	d	22°C	+	+

1 = Strain 6302/RB
2 = *E. cloacae* as described in the literature
(+) = Late positive reaction
d = Different

Table II summarizes the result of cross agglutination and cross absorption experiments. It is seen that living, alcoholized and heated *E. cloacae* 6302/RB gave high titre agglutinations in unabsorbed *Sh. sonnei* II serum (Exp. 1). Absorption of *Sh. sonnei* II serum with living or boiled 6302/RB decreased but did not remove the homologous titre (Exps 2 and 3). It should be noted that absorption with living culture was difficult and succeeded only after repeated experiments — this finding was due to an envelope antigen in strain 6302/RB.

Sh. sonnei II showed high titre agglutination in an immune serum prepared with living 6302/RB (Exp. 4). The much higher titre for the living homologous strain can be attributed to flagellar antibodies. O titre is indicated by agglutination obtained with heated bacteria. Absorption by *Sh. sonnei* II decreased the O titre (Exp. 5).

When serum 6302/RB (living) was absorbed with the heated homologous culture, living and alcoholized suspensions of 6302/RB still showed high titres

Table II

Antigenic analysis of E. cloacae 6302/RB and Sh. sonnei II

Immune sera: Designation	Immune sera: Absorption	Antigens: *Sh. sonnei* phase II	Antigens: *E. cloacae* 6302/RB living	Antigens: *E. cloacae* 6302/RB alcoholized	Antigens: *E. cloacae* 6302/RB 100°C	Experiment
Sh. sonnei	—	2560	1 280	320	640	1
phase II, living	6302/RB, living	320	—(1)	—	—	2
	6302/RB, 100°C	320	—	—	—	3
6302/RB, living	—	1280	20 480(2)	5120	2560	4
	Sh. sonnei II	—	5 120(2)	1280	640	5
	6302/RB, 100°C	—	2 560(3)	640	—	6
6302/RB,	—	420	10 240	5120	640	7
alcoholized	6302/RB, 100°C	—	1 280	1280	—	8
6302/RB, 100°C	—	640	5 120	5120	2560	9
	Sh. sonnei II	—	2 560	1280	640	10

Immunization and cross absorption were performed with cultures containing both L plus and L minus mutants.

(1) = less than 1 : 10

(2) = O and H agglutination

(3) = H and L agglutination

(Exp. 6). This finding indicates that strain 6302/RB contains thermolabile antigen and confirms that the antigen in 6302/RB related to *Sh. sonnei* II is an O factor (Exp. 7).

Agglutination of 6302/RB in unabsorbed and absorbed (Exp. 8) sera prepared with alcoholized culture shows that the organism contains an L-type surface antigen, which is not related to *Sh. sonnei* II.

Experiments 9 and 10 performed in 6302/RB serum prepared with heated bacteria confirm the O antigenic relationship to *Sh. sonnei* II.

As in the experiments there was no difference between the agglutination titres of living and heated *Sh. sonnei* II suspensions, only data obtained with heated bacteria are presented.

Discussion

Biochemical examination of strain 6302/RB showed that the organism belonged to *E. cloacae*. The species has been described as adonitol negative, although adonitol-fermenting strains have also been found [12]. Our strain fermented this substance and it is noticeable that it split lactose only after

3 days. The latter property was responsible for its shigelloid behaviour in course of the preliminary examination. The ONPG test, however, was positive in 30 minutes. The strain was flagellated, which is a characteristic property of *E. cloacae*.

Serological examinations indicated that strain 6302/RB contained O, K and H antigens. The K antigen was of the L-type as it was destroyed by heating. On subcultures the strain lost its K antigen. The loss of the surface antigen was accompanied by morphological alteration: the colonies became less convex and more translucent. The surface antigen was not related to *Sh. sonnei* II as OK serum 6302/RB failed to agglutinate this organism. As to *E. cloacae* K antigens only one report was available; according to EDWARDS and FIFE [14] these factors are related to *Klebsiella* capsular antigens.

Cross agglutination and cross absorption experiments indicated that an "ab—ac"-type relationship existed between the O antigens of strain 6302/RB and *Sh. sonnei* II; that is, in addition to the common component each organism contained a specific factor. In view of the antigenic relationship between *Sh. sonnei* II and *Sh. boydii* 6 and *E. coli* 014 as well as that between *Sh. boydii* 6 and *E. coli* 076 [15, 16], cross agglutinations with these antigens were also performed. There was a minor reciprocal relationship between strain 6302/RB and the above-listed *Shigella* and *E. coli* O antigens. It was also shown that strain 6302/RB and *Sh. sonnei* I contained no related antigens.

By slide agglutination in serum 6302/RB the behaviour of *Pseudomonas shigelloides*, $C_{27}S$ strain, $C_{27}R$ strain and *Plesiomonas shigelloides* was examined. These organisms belong to one species of doubtful taxonomic position. EWING *et al.* [17] classify them into *Aeromonas*, EDDY [18] to a new genus, *Plesiomonas*. These bacteria are serologically identical with *Sh. sonnei* phase I [19, 20]. RAUSS [21] observed that R mutants of these bacteria were identical in antigenic structure with *Sh. sonnei* phase II. As expected, *E. cloacae* 6302/RB showed a minor bilateral relationship to $C_{27}R$ mutant. In serum $C_{27}S$ strain 6302/RB showed no agglutination and vice versa, serum 6302/RB failed to agglutinate strain $C_{27}S$.

The present examinations indicate that, as emphasized by other authors [22—24], a simple serological examination is not sufficient for establishing the bacteriological diagnosis. Our studies show that even the combined use of simple biochemical tests and agglutination may lead to erroneous findings, namely strain 6302/RB behaved on triple sugar-urea-iron agar as shigellae and reacted in *Sh. sonnei* I—II serum. A similar finding was described by WINTON [24]. Accordingly, extended biochemical examination or certain specific tests should be performed not only with atypical but also with seemingly well-recognizable organisms.

E. cloacae strain 6302/RB and the serum prepared from it may be useful in checking *Sh. sonnei* I and *Sh. sonnei* II strains and sera.

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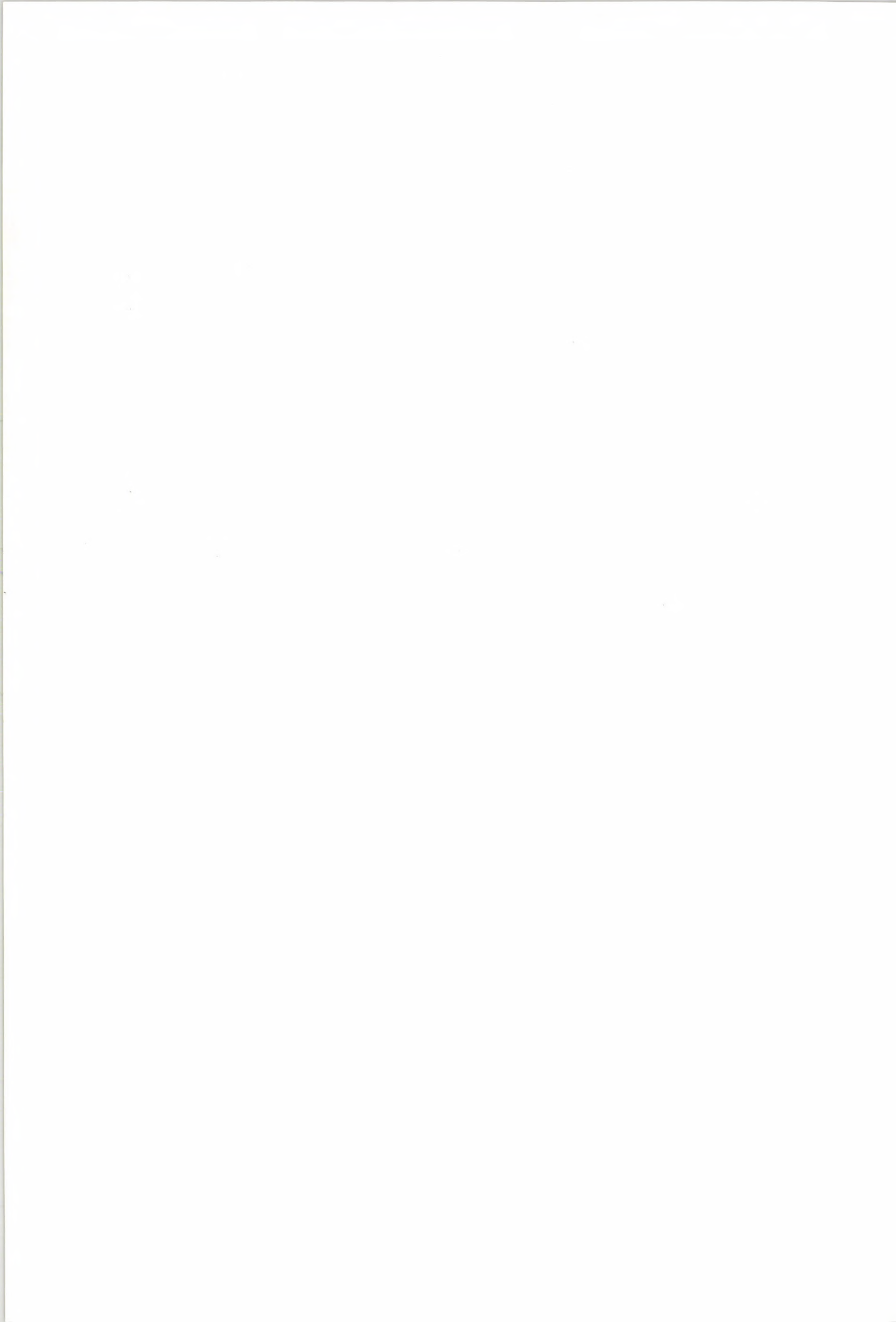

Acta Microbiol. Acad. Sci. hung. **16,** *309—317, 1969*

PHAGE AND COLICIN TYPING OF SHIGELLA SONNEI

By

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(Received April 17, 1969)

Summary. Phage typing of 3522 strains and colicin typing on the basis of colicinogenicity of 1418 strains of *Shigella sonnei* isolated in the years 1966—68 from various parts of Hungary have been performed. The types were stable on repeated examinations *in vivo* and *in vitro* and after conjunctival passage in the guinea pig.

Phage types 6, 65 and 7 occurred most frequently. Of colicin types 0, 6, 2 and 4 showed the highest incidence.

By colicin typing various phage types were subdivided. The combined use of the two methods was found suitable for epidemiological investigations.

Dysentery comprises an important public health problem all over the world. For epidemiological investigations it is necessary to divide the causative agent into smaller units. The increasing incidence of *Shigella sonnei* which cannot be subdivided serologically, made the elaboration of methods suitable for further differentiation necessary. This problem has been solved by elucidating the specific phage and colicin sensitivity and colicinogenic properties of *Sh. sonnei.*

Materials and methods

Sh. sonnei strains were collected from various areas of Hungary in the years 1966—68. The strains were isolated from dysenteric patients and carriers.

Phage typing was performed by HAMMARSTRÖM's method [1]. After propagation the routine typing dilution was determined for each of the 12 phages. Typing was performed as follows. Several R colonies were picked up from agar plate and inoculated into broth. After 2 hours incubation at 37°C the culture was seeded onto agar medium, then the phages were spotted on the plates. Readings were made after incubation at 30°C overnight.

Colicin production and type determination were carried out by the modified method of ABBOT and SHANNON [2]. A 24-hour broth culture was prepared from each *Sh. sonnei* strain and spotted on 9 different agar plates so that each plate was inoculated with 6 different strains. After 16 hours incubation at 37°C the plates were exposed to chloroform vapour for 30 minutes, then left to stand with open lid for 5 minutes. Finally 2-hour broth cultures of the 9 indicator strains were mixed into soft agar (0.5 ml to 4 ml medium) and layered over the plates. Inhibition zones were read after incubation at 37°C overnight. Colicin types were determined as shown in Table I [3]. GILLIES and BROWN [4] supplemented the colicin typing scheme with two new types.

The ability of the strains to cause keratoconjunctivitis in guinea pigs was examined as described by SERÉNY [5].

Results

A total of 3522 *Sh. sonnei* strains were phage typed. The strains belonged to 28 different phage types. Untypeable or degraded cultures comprised only 5.4%. Distribution according to phage types is presented in Table II.

The most frequently occurring three phage types were 6, 65 and 7. Ten of the phage types were encountered in 0.5—4.8%, fifteen of them were isolated but occasionally.

Table I

Inhibition patterns of Sh. sonnei colicin types

Colicin type	Indicator strains								
	56	17	56/56	2	R6	M19	2/7	R5	Row
1A	+	+	+	+	—	+	—	—	+
1B	+	+	+	+	+	+	—	—	+
2	+	+	—	—	+	—	—	—	+
3	+	+	+	+	+	+	+	+	+
3A	+	+	+	+	+	—	+	+	+
4	+	+	—	+	+	—	+	—	+
5	+	+	+	+	+	+	+	—	+
6	+	—	+	—	+	—	—	—	+
7	—	+	—	—	—	—	—	—	—
8	—	+	—	+	—	—	—	—	+
9	+	+	+	+	+	—	—	—	+
10	+	+	+	+	+	+	—	—	+
11	—	—	—	—	—	—	—	—	+
12	+	—	+	+	+	—	—	—	+
13	+	+	+	—	+	—	—	—	+
0	—	—	—	—	—	—	—	—	—

+ = inhibition
— = no inhibition

Colicinogenicity of *Sh. sonnei* isolates is shown in Table III.

Forty-nine per cent of the examined 1418 strains were colicinogenic and, on the basis of colicin produced, fell into 14 types. Colicin types 6, 2 and 4 occurred relatively very frequently. Strains not producing colicin were regarded as a separate group and were designated according to HART's nomenclature [3] as O.

Different phage types were subdivided according to their colicin types: Table IVa shows the distribution of the three frequent phage types.

Table II
Phage type distribution of Sh. sonnei strains
(According to HAMMARSTRÖM's scheme)

Phage type	Isolates	
	Number	Per cent
2	51	1.4
3	56	1.6
5	4	0.1
6	1137	32.2
7	411	11.7
8	7	0.2
12	168	4.8
13	124	3.5
15	4	0.1
17	13	0.4
19	28	0.9
20	7	0.2
23	1	0.1
24	13	0.4
25	10	0.3
26	—	—
30	12	0.3
31	7	0.2
32	49	1.4
34	4	0.1
37	12	0.3
39	—	—
41	28	0.9
42	4	0.1
55	4	0.1
61	43	1.2
62	83	2.4
63	—	—
65	1015	28.8
67	—	—
68	23	0.5
86	—	—
94	12	0.3
Degraded	126	3.6
Untypeable	66	1.9
Total	3522	100.0

Table III

Colicin type distribution of Sh. sonnei strains

Colicin type	Isolates	
	Number	Per cent
1B	1	0.1
2	164	11.6
3	15	1.0
3A	4	0.3
4	104	7.3
5	1	0.1
6	360	25.4
7	2	0.1
8	8	0.6
9	2	0.1
11	15	1.1
12	13	0.9
13	10	0.7
15	1	0.1
Degraded	2	0.1
0	716	50.5
Total	1418	100.0

Table IVa

Distribution of frequent phage types of Sh. sonnei according to colicin types

Phage type	Colicin types encountered	Predominant colicin type and number of strains		Total number of strains
6	2, 3A, 4, 6, 7, 8, 11, 12, 0	0	242	273
		2	13	
7	2, 3, 4, 5, 6, 11, 0, degraded	4	59	107
		0	32	
		2	8	
65	2, 6, 11, 12, 13	6	81	87

Table IVb

Distribution of frequent colicin types of Sh. sonnei according to phage types

Colicin type	Phage types encountered	Predominant phage type and number of strains		Total number of strains
2	2, 3, 6, 7, 55,	6	13	37
	61, 65, degraded	7	8	
4	3, 6, 7, 12, 13, 19, 20,	7	59	93
	25, 31, 32, nt	3	19	
6	2, 6, 7, 12, 13,			
	17, 19, 25, 37, 61,	65	81	118
	65, 68, degraded			
0	2, 3, 5, 6, 7, 8,	6	242	
	12, 13, 15, 19, 24,	12	47	388
	25, 30, 31, 34, 37,	7	32	
	55, degraded, nt	2	21	

Within the phage types certain colicin types predominated but there was no strict association between phage and colicin types. In Table IVb phage types are grouped according to frequent colicin types.

Table V

Phage and colicin types of Sh. sonnei strains isolated on repeated examination of the same person

Phage type	Colicin type	Number of persons	Number of examinations
3	4	1	2
3	0	1	2
6	2	2	6
6	0	6	12
7	4	3	6
7—3	4	1	2
12	4	1	2
12	12	1	2
37	0	1	3
61	6	5	10
61—6—61	6	1	3

Epidemiological data indicated that the subdivision of phage types by colicin types is justified, as each defined focus was represented by strains belonging to one type.

In order to check the stability of phage and colicin types, strains isolated from the same person on repeated examinations, from family and community outbreaks, were studied. Model experiments using guinea pig conjunctival passage were also performed.

Table V presents the phage and colicin types of isolates obtained on repeated examinations. As compared to the first isolation, in 21 persons there was no change in phage and colicin types; from 2 persons the same colicin types but different phage types were cultured.

Table VI

Phage and colicin types of Sh. sonnei strains isolated from family outbreaks

Phage type	Colicin type	Number of families
2	0	2
3	4	1
6	0	7
7	4	7
12	0	1
19	6	1
30	0	1
65	6	8
Total		28

A total of 28 family outbreaks were examined. Members of the same family excreted always the same phage and colicin type (Table VI).

Similar results were obtained in communities. Four outbreaks were associated with phage type 6, non-colicin-producing strains. For 2 outbreaks phage type 65 colicin type 6 strains were responsible. In one community 10 children excreted phage type 12, non-colicinogenic, 1 child phage type 37, non-colicinogenic *Sh. sonnei.*

Stability examinations by conjunctival passage in guinea pigs yielded the following results. Thirty-six strains had been isolated from one outbreak; colicin typing showed them to belong to type 2, but according to phage typing 2 of them belonged to type 13 and the rest to type 6. From sporadic cases 15 strains were examined (phage types 65, 32, 12, 7, 6 and degraded; colicin

types 12, 6, 2 and 0). Through 3 passages there was no change in colicin types in any of the strains. According to phage typing one strain (type 6) became degraded after the first conjunctival passage.

Discussion

A method for the phage typing of *Sh. sonnei* was elaborated by HAMMARSTRÖM in 1949 [1]. Although the method may not yield very stable results, it is useful for practical purposes. Attempts to standardize the technique are in progress [6].

Independently from HAMMARSTRÖM another method for *Sh. sonnei* phage typing has been described by TEE [7], who divided 829 strains into 20 types with 10 phages. GROMKOVA and TRIFONOVA [8] prepared two sets of phages for typing S and R forms and found that by both methods 65—75% of the examined strains fell into one phage type.

HAMMARSTRÖM'S phages were used by many authors. In England MAYR-HARTING [9], in France SZTURM-RUBINSTEN [10, 11], in Australia LUDFORD [12], in Czechoslovakia RAŠKA *et al.* [13], in Poland KUCHAREWICZ [14], in East Germany RISCHE [15, 16], JUNGHANS [17] and GRUNOW [18] demonstrated that the method was suitable for epidemiological investigations.

In course of basic systematic and genetic studies on colicins FREDERICQ [19—21] was the first to use colicin sensitivity for epidemiological studies in *Salmonella* and *Shigella* infections. ABBOT and SHANNON [2] were inspired by these studies to investigate the colicin sensitivity of *Sh. sonnei.* They showed that strains from the same source were different in colicin sensitivity. As they found colicin production a stable genetic property, they concluded that colicinogenicity was more suitable for typing than colicin sensitivity. In examining colicin production by 537 strains they were able to type 68% of the cultures; 32% of the strains produced no colicin. Typing by this method gave satisfactory results in epidemiological studies. This finding has been confirmed by HART [3]. The usefulness of colicin production as an epidemiological marker has also been pointed out by GILLIES [22].

There are data for the alteration of *Sh. sonnei* phage types. MAYR-HARTING [9] described that typing by HAMMARSTRÖM'S set of phages revealed occasionally more than one phage type from the same outbreak. She was able to alter the phage type of the strains by exposing them to the effect of phages *in vitro.* From her results she concluded that changes in phage type may occur also *in vivo.*

RISCHE [16] reported on the incidence of *Sh. sonnei* phage types in different countries. Type 3 occurred most frequently in Czechoslovakia, England and Poland, type 8 in France, types 3, 5 and 13 in East Germany and types

65, 12 and 13 in Berlin. According to KALLINGS *et al.* [6] in Sweden types 5 (25%) and 3 (22%) predominated; the incidence of types 7, 62 and 65 was higher than 5%.

The incidence of colicin types also varies according to countries and districts. HART [3] described that in England types 7 and 0, in other countries types 0, 6 and 12 were most frequently isolated. In Scotland GILLIES [22] studied the distribution of colicin types over a period of 5 years. In the beginning type 7 predominated, later a gradual increase in the number of non-colicinogenic strains was observed, while in a different area the predominating untypable strains were replaced gradually by type 7.

Our results confirm data that phage typing and colicin typing are both suitable for epidemiological investigations. Strains isolated from one focus were identical in phage and colicin types. Repeated examinations *in vivo* and *in vitro* also showed the stability of phage and colicin types. There was no alteration in course of passaging the strains by conjunctival infection in guinea pigs.

Repeated examinations revealed change in phage type on two occasions. One strain showed alteration during conjunctival passage. These infrequent alterations do not diminish the practical value of phage typing.

Hungary differed from other countries in respect to the distribution of *Sh. sonnei* phage and colicin types. Of types predominating in Hungary (6, 65 and 7) only type 65 occurred in great number in Berlin in 1955. There were no available data about phage types 6 and 7 found frequently in Hungary. Phage types 3 and 5 occurring commonly in other countries were encountered in Hungary only in 1.6 and 0.1%, respectively.

According to colicin types, in agreement with data for other countries, types 0 was the commonest. Types 6 and 4 were also frequently met with.

Combined phage and colicin typing has been shown suitable for subdividing the frequent types and thus for epidemiological investigations.

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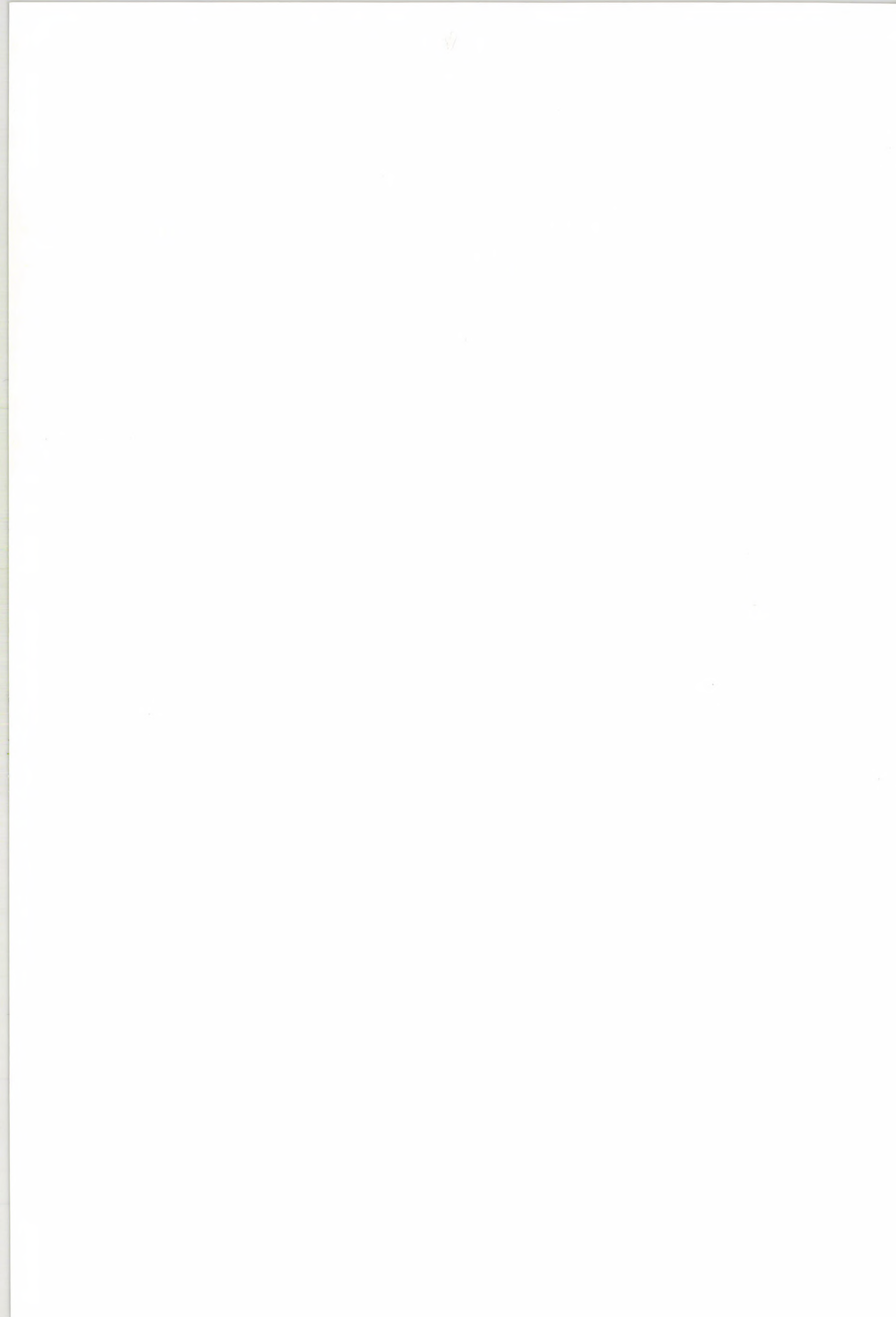

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INTERFERON STIMULATION AND OTHER PHENOTYPIC PROPERTIES OF TWO VARIANTS OF POLYOMA VIRUS

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Summary. Two polyoma virus strains, Toronto-26 (T-26) and SE-55, were shown to differ not only in their oncogenic effect on the newborn hamsters, but also in several other properties, which were demonstrated *in vitro* in embryonic mouse and hamster cells.

T-26, the strain showing the more pronounced oncogenic activity, stimulated interferon production in embryonic mouse and hamster cells more rapidly and in a much higher titre compared to the strain SE-55, which had a lower oncogenic activity.

Further to this, in embryonic mouse cell culture the strain T-26 showed a slower synthesis of infective virus and slower development of cytopathic effect, as well as a higher haemagglutinating activity, as contrasted to the strain SE-55.

The relation between the oncogenic and interferon stimulating activities of the examined polyoma virus variants is discussed.

Some variants of polyoma virus differ in their oncogenic properties for the mouse and hamster [1—4] as well as in various other properties demonstrable in cell culture, such as plaque size [5—7], proportion of transformed clones [8], rate of cytopathic effect [9], and sensitivity to bicarbonate concentration [10].

It was demonstrated in mouse experiments that polyoma virus mutants of different oncogenic activity stimulate interferon in mouse cell cultures in different degrees [11, 12].

In this laboratory, two strains of polyoma virus, T-26 and SE-55, which differ in oncogenity for the hamster, were studied. Attempts were made to compare their phenotypic properties including interferon stimulation in embryonic mouse and hamster cell cultures.

Materials and methods

Virus strains. The Toronto strain of polyoma virus [13] was supplied by Professor M. P. CHUMAKOV (Institute for Poliomyelitis and Viral Encephalitides, Moscow) and prior to use was carried through 10 passages in embryonic mouse cells in the laboratory.

The SE-55 polyoma virus strain [4] was supplied by Dr. I. HELLSTRÖM (Karolinska Institutet, Stockholm) and was carried through 12 passages in embryonic mouse cell culture.

In our experiments, the above strains were designated as T-26 and SE-55, respectively.

The Semliki Forest Virus [14] strain and Chikungunya virus [15], received from the Institute of Poliomyelitis and Virus Encephalitides, Moscow, were carried through 5 intracerebral passages in BALB mice. As virus material, a 20% mouse brain suspension was used. Prior to use, the material was centrifuged at 2500 r.p.m. for 30 minutes and the sediment was discarded.

Cell cultures. Mouse, hamster and chick embryo cell cultures, as well as cultured cells from the peritoneal exudate of mouse and hamster were used.

The monolayer cultures of embryonic mouse and hamster cells were prepared from embryos removed a few days prior to birth. The minced tissue was washed with saline and treated with 0.1% trypsin solution in flasks at 37°C for 30 minutes. Subsequently, the trypsin solution was discarded and the minced tissue was pressed with a glass rod through 4 layers of gauze mounted on a glass funnel and occasionally washed with nutrient medium. After counting and appropriate dilution, the cell suspension was distributed into test tubes or 1 litre Roux flasks (300,000 cells/ml).

Cultures of embryonic mouse and hamster cells were prepared with Parker's medium No. 199 and Eagle solutions, containing 10—15% of calf serum. Chick embryo cell cultures were prepared in a similar way from 9—10 days old embryos. As a growth medium, Hanks' solution containing 5% cattle serum and 0.5% lactalbumin hydrolysate, was used.

Cell cultures from the peritoneal exudate of the hamster were prepared as follows. One or 2 ml of a 2% solution of starch in saline was injected into the peritoneal cavity of the hamster. One, two, or three days later 2—5 ml of Parker's medium No. 199 containing 30% calf and 10 I.U./ml of heparin, was injected by the same route. After a slight abdominal massage, the peritoneal exudate cells were withdrawn with a syringe and after counting they were made up to a concentration of 3—5 million/ml in 30% calf serum containing Parker's medium No. 199 and distributed into tubes.

In certain cases, the peritoneal cell culture was prepared without preceding injection of starch.

Virus titration. The titre of the Semliki Forest Virus and polyoma virus was assessed *in vitro*, on the basis of its cytopathic effect in primary cell cultures from mouse embryos of the BALB strain. The virus dilutions were inoculated into 6 tube cultures each.

Chikungunya virus was titrated in primary embryonic hamster cell culture, and the cytopathic effect was read. Titrations were carried out also in chick embryo cell culture by plaque titration with the agar overlay method, using 100 ml stoppered flasks containing a confluent monolayer of chick embryo cells. Prior to the addition of the virus, the cell culture was washed once with saline. Subsequently, tenfold serial dilutions of the virus were inoculated each into 2—3 flasks in 0.3 ml amounts. The virus was allowed to adsorb at 37°C for 1—1.5 hours, then the cultures were covered with nutrient medium containing agar and placed in an incubator at 37°C. The results were read after 3—4 days.

Haemagglutination. The polyoma virus strains were titrated for HA activity in plexiglass plates provided with rows of wells to hold the reactants. Twofold serial dilutions of the virus were prepared with a 15 *N* K—Na-phosphate buffer pH 8.5. 0.5 ml of each dilution was placed in a well in succession. Subsequently, 0.15 ml of a 3 times washed 1% guinea-pig erythrocyte suspension was added to each dilution. The plates were incubated overnight at 4°C. Reactions were read with the four-cross system, the titre was expressed as the highest dilution still giving a three- or four-cross agglutination.

In vitro production of interferon with polyoma virus. Cultured embryonic cells from BALB mice or gold hamsters were inoculated with polyoma virus (the doses are given in the chapter "Results"). The inoculated cultures were incubated for 2 hours at 37°C, washed 3 times and then Parker's No. 199 medium containing 2% cattle serum was added. The haemagglutinating and infective titres of the nutrient medium were determined at different intervals, then the virus was removed by appropriate treatment and the material was titrated for interferon.

To remove polyoma virus prior to titration for interferon the fluid media were dialysed against pH 2.0 KCl—HCl buffer for 72 hours at 4°C. Subsequently the pH was adjusted to 7.5 by dialysis against Earle's solution.

In certain cases, the material was centrifuged at 100,000 *g* for 2 hours prior to the determination of interferon content.

Titration of interferon. Mouse interferon was titrated by the CPE-inhibition method [16]. The test materials were diluted in Parker's medium No. 199 containing 2% cattle serum, inoculated into primary embryonic BALB mouse cell cultures previously washed with Hanks' solution, and incubation for 4 hours at 37°C. Each dilution was inoculated into at least 4 tube cultures. After incubation the cell culture was washed 3 times with Hanks' solution and infected with 50—100 CPD_{50} of Semliki Forest Virus. As controls, the latter virus and known mouse interferon were titrated in parallel. Results were read on the 3rd—4th day and related to the complete cell degeneration in the interferon-free virus-infected cultures as controls. The titre of interferon — in nutrient medium or mouse serum — was expressed in terms of the highest dilution still inhibiting the cytopathic effect of Semliki Forest Virus in 50% of the tube cultures.

The interferon formed in cultured embryonic or peritoneal hamster cells was titrated by the virus synthesis inhibition method [17, 18]. The materials were inoculated into primary embryonic hamster cell cultures and incubated for 4 hours at 37°C. Subsequently, the cultures were washed 3 times and superinfected with 50—100 PFU/0.2 ml of Chikungunya virus. The cultures were incubated at 37°C for 1.0—1.5 hours to allow the virus to adsorb, then they were washed 3 times to remove the virus and Parker's No. 199 medium containing 2% cattle serum was added as nutrient medium. With all experiments, the following control series were set up: (1) virus titration; (2) titration of interferon of known titre.

Eighteen hours later the infected cultures were three times frozen and thawed at —70°C and +37°C, respectively. The total quantity of Chikungunya virus was determined in chick embryo cell culture by plaque titration with the agar overlay method.

The titre of interferon was expressed in terms of the highest dilution of nutrient medium which still caused 50% inhibition of Chikungunya virus synthesis as related to the controls.

All comparative examinations of interferon were carried out simultaneously on one and the same batch of cell culture.

Determination of the molecular weight of interferon. The molecular weight of the inhibitor induced in embryonic hamster cell culture with polyoma virus was determined by means of an LKB fraction collector by chromatography on a Sephadex G-100 column.

Elution was carried out with 0.1 *M* phosphate buffer, pH 7.2, at a rate of 25 ml/hour. Prior to the measurements, the Sephadex G-100 column was calibrated with proteins of known molecular weight.

The highest biological activity of the inhibitor was demonstrated in the fractions of total volumes between 232.4—243.4 ml which corresponded to molecular weights of 21,500 and 24,000, respectively [19].

Results

(A) *Oncogenic properties of the two polyoma virus variants.* The polyoma virus strain T-26 caused visceral tumours, especially renal sarcoma, in 100% of the cases when given to newborn hamsters on their first day of life. An identical dose, 10^4 CPD_{50} subcutaneously, of strain SE-55 caused neoplastic changes, mainly subcutaneous sarcoma, in 71% of the cases. The two polyoma virus variants differed not only in their oncogenic effect but also in respect of the latency period preceding the development of the tumour, the time of death and the development of haemorrhages (Table I).

Table I

Characterization of the oncogenic process induced in hamsters with two variants of polyoma virus

(Infection: 1st day after birth)

Virus strain	Dose of virus (log CPD_{50} 0.2 ml)	Incidence of				Time elapsed before appearance of tumour (weeks)	Survival (weeks)
		Tumour formation	Renal sarcoma formation	Subcutaneous sarcoma formation	Haemorrhages		
T-26	4.0	50/50*	50/50	0/50	48/50	1—2	2— 3
SE-55	4.0	48/68	6/48	25/48	10/48	4—6	6—12

* Numerator: Number of animals with tumour
Denominator: Number of infected animals

After inoculation of strain T-26, the kidneys soon showed a gross enlargement, but hardly any change of shape. Microscopically, a diffuse enlargement of both the cortex and medulla was found. The renal tumours were identified as infiltrating fibrosarcoma. The tumour and the haemorrhages were as a rule well visible at necropsy during the first week. All hamsters infected with the T-26 strain died in 2 or 3 weeks. Infection of hamsters with strain T-26 at the age of 1 or 2 weeks also resulted in 100% lethality. But the life span of the infected animals was prolonged by 4 weeks, renal sarcoma developed in 52% of the cases only, while the haemorrhages were pronounced. The haemorrhagic cysts located beneath the renal capsule often reached 1 cm in diameter.

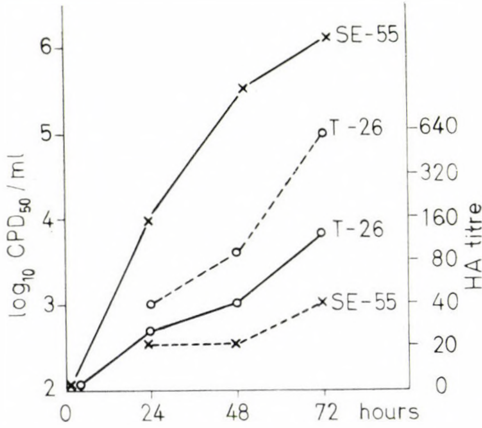

Fig. 1. Accumulation of infective and haemagglutinating virus in nutrient medium of cultured embryonic mouse cells (BALB line)
Infective titre of virus: T-26 ○——○; SE-55 ×——×. HA titre: T-26 ○------○; SE-55 ×------×

(B) *Growth of polyoma virus in embryonic mouse cell culture.* Other properties of the two polyoma virus variants were studied in embryonic mouse cell cultures. The two strains were found to differ in the production of infective virions, the rate of development of cytopathic effect and in their haemagglutinin-synthetizing capacity.

Fig. 1 shows average values for three experiments. Accumulation of infective virions in the nutrient media of BALB embryonic mouse cell cultures was slower with the T-26 than with the SE-55 strain. The infective titre of the two variants differed after 48 hours by approximately 3 logarithmic orders, while after 72 hours by 2 logarithmic orders (Table II).

Both polyoma virus strains caused nearly total cell degeneration in the infected embryonic mouse cell cultures. The cytopathic effect of strain T-26 developed much slower than that of strain SE-55. Accordingly, the final CPE titre of strain T-26 could not be read until the 21st day after infection (Table III), while that of strain SE-55 was read on the 14th day.

Table II

Infective and haemagglutinating titres of polyoma virus in embryonic mouse cell culture at different intervals after infection

Experiment No.	Virus strain	Multiplicity (CPD_{50}/cell)	Virus titre									
			Log_{10} CPD_{50}/ml					Haemagglutination				
			Days					Days				
			3	5	7	9	14	3	5	7	9	14
1	T-26	5	3.7	4.2	5.5	6.3	6.5	640	640	1280	1280	1280
	SE-55	5	5.9	6.2	6.3	7.0	7.0	40	80	80	180	320
2	T-26	5	2.8	3.3	4.5	5.0	5.8	160	320	320	640	640
	SE-55	5	4.2	4.5	5.9	6.2	6.3	20	40	80	80	160
3	T-26	1	2.5	3.3	3.8	5.0	5.8	160	320	640	1280	2560
	SE-55	1	4.7	4.9	5.3	6.0	6.0	40	80	160	160	320

Table III

Titration of polyoma virus variants on the basis of CPE in BALB embryonic mouse cell culture

Experiment No.	Virus strain	Titre (log_{10} CPD_{50}/ml) on days				
		5th	10th	14th	17th	21st
1	T-26	2.65	3.70	5.32	5.85	6.37
	SE-55	3.10	5.83	6.10	6.10	6.10
2	T-26	2.95	4.10	4.85	5.30	6.10
	SE-55	3.70	5.20	5.85	5.85	5.85
3	T-26	3.65	4.80	5.57	6.10	6.50
	SE-55	4.00	5.72	6.28	6.28	6.28

(C) *Induction of interferon by polyoma virus in embryonic mouse cell culture.* Stimulation of interferon by polyoma virus was examined in embryonic BALB mouse cell cultures, where both polyoma virus variants induced interferon production.

Increase in the infective titre and interferon formation is shown in Table IV as observed from 4 experiments. In embryonic mouse cell cultures, strain T-26 and SE-55 did not differ notably in titre on the 14th day after infection. The interferon titre, however, was 4—8 times higher in the cultures infected with T-26 than in those infected with SE-55.

Interferon accumulation took place slower on stimulation with SE-55 than with T-26; in the former case, signs indicative of the presence of interferon were not observed until the 9th day. At the same time, the interferon induced by strain T-26 was already demonstrable on the 4th day.

(D) *Induction of interferon by polyoma virus in embryonic hamster cell culture.* No literary data were found concerning interferon stimulation by polyoma virus in embryonic hamster cell culture. It seemed therefore interesting to examine the induction of interferon in that type of cell culture by two polyoma virus variants which differed in oncogenicity for the hamster, and to seek a correlation between the oncogenic and interferon stimulating properties of these strains. All infection experiments were performed on newly prepared primary embryonic hamster cell cultures. These showed a considerable indi-

Table IV

Infective titre of polyoma virus and interferon titre in embryonic mouse cell culture at different intervals after infection

Experiment No.	Virus strain	Infectious dose (log_{10} CPD_{50}/ml)	Virus titre (log_{10} CPD_{50}/ml)			Interferon titre		
			Days			Days		
			4	9	14	4	9	14
1	T-26	6.3	4.0	5.3	6.0	2	8	32
	SE-55	6.7	5.5	5.8	6.3	0	0	4
2	T-26	6.3	4.2	5.0	6.5	4	8	32
	SE-55	6.7	5.8	6.0	6.8	0	2	8
3	T-26	5.8	3.8	5.0	6.8	4	16	64
	SE-55	6.0	5.8	6.5	7.0	0	2	8
4	T-26	5.8	N.E.	N.E.	6.3	8	16	64
	SE-55	6.0	N.E.	N.E.	6.0	0	4	16

N.E. = not examined

vidual variation of sensitivity to polyoma virus, though embryos from the same mother animal were used for each lot of tissue culture.

In part of the cultures, there was an increase of the haemagglutinating and infective titre, while in 2 of 5 cultures no synthesis of polyoma virus was demonstrable. In the embryonic hamster cell culture, replication of the two polyoma virus variants was not accompanied by a visible cytopathic effect.

Regardless of the individual variation in the infective virus synthesizing ability of primary embryonic hamster cell cultures, it was clearly demonstrable

that infection with polyoma virus was followed by the formation of a virus inhibitor substance. In 3 of 5 primary embryonic hamster cell cultures, the inhibitor had formed parallel to the increase of the haemagglutinating and infective virus titres (Fig. 2), while in the remaining 2 cultures there was inhibitor formation but no evidence of the synthesis of infective or haemagglutinating virus (Fig. 3).

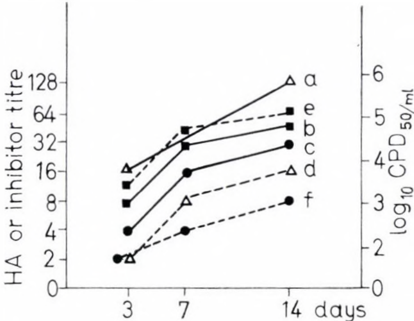

Fig. 2. Stimulation of inhibitor formation by polyoma virus in embryonic hamster cell culture (Experiment 1). Explanations: a: HA titre; b: infective titre; c: inhibitor titre in the case of strain T-26; d: HA titre; e: infective titre; f: inhibitor titre in the case of SE-55 strain

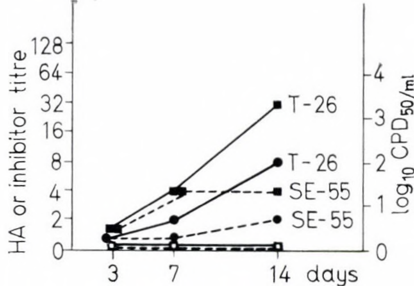

Fig. 3. Stimulation of inhibitor by polyoma virus in embryonic hamster cell culture (Experiments 2 and 5). Designations: ■ Titre of infective virus; ● Titre of inhibitor (Experiment 2); ▣ Titre of inhibitor (Experiment 5)

Pretreatment of embryonic hamster cell cultures with nutrient medium from the non-infected control tubes had no influence on Chikungunya virus yield. Chikungunya virus synthesis was inhibited exclusively by the inhibitor substance which was stimulated by polyoma virus.

The results obtained in embryonic hamster cells agreed well with those found in embryonic mouse cells, in other words the T-26 strain of stronger oncogenicity, stimulated a more rapid, and quantitatively higher synthesis of inhibitor substance than did the strain SE-55.

(E) *Properties of the interferon induced by polyoma virus in embryonic hamster cell cultures.* Some biological and physico-chemical properties of the

virus inhibitor induced by polyoma virus on embryonic hamster cell cultures were examined in lack of relevant literary data. The cell specificity and virus specificity of the inhibitor, the effects on its titre of the pH and of dialysis, its sensitivity to trypsin, RNAse, DNAse and anti-polyoma serum, as well as its ultracentrifugal sedimentation, actinomycin D effect and molecular weight were determined (Table V).

On the basis of its biological and physico-chemical properties, the viral growth inhibitor stimulated in embryonic hamster cell culture by polyoma virus has been regarded in interferon-like protein.

Table V

Biological, physical and chemical properties of the interferon induced by polyoma virus in embryonic hamster cell culture

1. Species specific
2. Not specific for virus
3. Stable at pH 2.0 for 5 days
4. Non-dialysable against buffer for 5 days
5. Not sedimented by ultracentrifugation at 100,000 g for 2 hours
6. Molecular weight 21,500—24,000
7. Trypsin-sensitive (on exposure to 200 μg/ml for 3 hours at 37°C)
8. Not sensitive to RNAse or DNAse treatment (100 μg/ml, 3 hours at 37°C)
9. Not neutralisable by specific polyoma antiserum
10. Inhibitory action blocked by actinomycin D (100 μg/ml)

(F) *Induction of interferon by polyoma virus in cultured cells from the hamster's peritoneal exudate.* Hamsters were given intraperitoneally a 2% starch solution and cells were collected from the exudate on the 1st—3rd days. Prior to the injection of starch, the hamster's peritoneal exudate consisted chiefly of cells with lobed nuclei, some macrophages and a few lymphocytes. After the injection of starch, the large macrophages tended to increase in number until they had made up 80% of the total cell count by the 4th day (Table VI).

The exudate cells were suspended at a concentration of 3—5 million cells/ml and cultured in tubes. They had a very low mitotic activity and seemed to form a confluent monolayer but the outgrowth essentially consisted of single independent cells situated closely to each other. The culture could be maintained for 1 or 2 months, then the round macrophages underwent a fibroblast-like transformation and formed growing monolayer islands.

Polyoma virus added 2—3 days after the adherence of the cells to the tube wall produced no distinct cytopathic effect. Three–five days after infection there was a certain rise in the haemagglutinating activity of the nutrient medium.

Table VI

Proportions of cellular elements in the hamster's peritoneal exudate before and after mobilization with starch

Cellular elements	Percentual distribution in the				
	0	24th	48th	72nd	96th
	hour after mobilization with starch*				
Macrophage	12.5	16.8	39.8	75.0	79.2
Lymphocyte	5.0	6.8	18.8	17.0	17.4
Cells with lobed nuclei	82.5	76.4	41.4	8.0	3.4

* Related to 1000 cells

In the nutrient medium of the cultured peritoneal exudate cells infected with polyoma virus, the presence of a substance inhibiting the growth of Chikungunya virus was demonstrated. In the embryonic hamster cell cultures

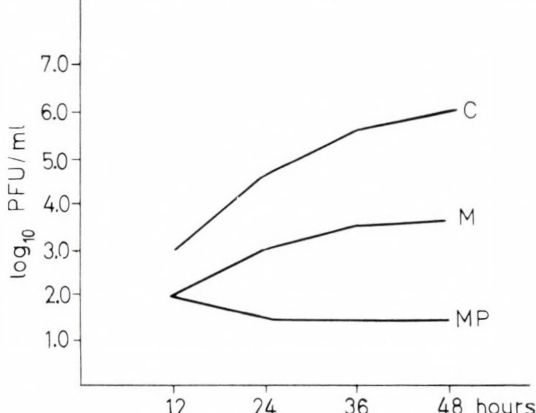

Fig. 4. Chikungunya virus synthesis in embryonic hamster cell cultures untreated (C) and treated with fluid medium from non-infected (M) or polyoma virus infected (MP) macrophage cultures

previously treated with nutrient medium from the infected macrophage culture, no Chikungunya virus synthesis took place (Fig. 4, curve MP). The inhibitory titre of the macrophage culture fluid rose parallel with its haemagglutinating titre. The inhibitory substance induced by polyoma virus was present in all macrophage cultures, regardless of the time of collection of the cells from the peritoneal exudate.

Based on its properties, the inhibitor can be classified as an interferon-like substance. It was inactivated by trypsin treatment, but its titre remained unchanged on treatment with anti-nuclease or anti-polyoma virus sera, ultracentrifugation and dialysis.

Closer studies of the inhibitory substance formed on polyoma virus infection of cultured hamster peritoneal exudate cells showed that a substance partially inhibiting the synthesis of Chikungunya virus was present also in the nutrient medium of non-infected macrophage cultures (Fig. 4, curve M). The presence of this substance was demonstrable also in peritoneal cell cultures obtained without preceding mobilization with starch, as well as in those cultures which were prepared 1—2, or 3—4 days after the injection of starch. During macrophage cultivation, there was a slight elevation in the inhibitor's activity.

Inhibition of the growth of Chikungunya virus was caused also by nutrient medium from cells stored at +4°C for 24 hours as well as by that from cells deep-frozen immediately after their withdrawal from the peritoneal cavity (Table VII).

Table VII

Influence on Chikungunya virus synthesis of interferon from non-infected peritoneal exudate cells of hamster

Treatment of peritoneal exudate cells	Synthesis of Chikungunya virus as related to controls, per cent	Interferon titre in undiluted nutrient medium
Cultivation for 24 hours	0.2—10	1—2
Cultivation for 5 days	0.2— 8	2—4
Stored at +4°C for 24 hours without culturing	0.1— 1	1—2
Stored at —20°C without culturing	10 —12	1

The properties of the inhibitor substance were studied. The inhibitor obtained from noni-nfected peritoneal exudate cells was sensitive to trypsin, insensitive to ultracentrifugation and dialysis, but previous actinomycin D treatment of the cells blocked the inhibitor's activity in the embryonic hamster cell culture.

Table VIII

Effect of actinomycin D on the inhibitor's activity

Pretreatment of cell culture	Titre of Chikungunya virus, PFU/0.3 ml
1. No pretreatment	2×10^2
2. Actinomycin D (1 μg/ml)	3×10^2
3. Inhibitor (titre 1 : 8)	0
4. Actinomycin D + inhibitor	4×10^2

The above outlined properties of the inhibitor obtained from non-infected hamster peritoneal exudate cell cultures, and, primarily, the blocking of its action by actinomycin D (Table VIII), in other words its relationship with DNA-dependent RNA synthesis, allowed the classification of the inhibitor into the group of interferon-like substances.

Discussion

So far, only few authors have studied the role of interferon in the virus-induced malignant transformation of cells. It has been reported that exogenous interferon had a direct inhibitory effect on cellular transformation processes initiated by SV-40 [20] or Rous sarcoma virus [21] *in vitro*, while *in vivo* it inhibited the neoplastic activity of polyoma virus [22], FRIEND's mouse leukaemia virus [23, 24] and the formation of T (tumour) antigen in SV-40-transformed cells [25]. In cell systems enhancing viral growth, exogenous interferons reduced viral tumour genesis by inhibiting virus synthesis [26, 27, 28].

It was demonstrated that interferon inhibits the translation of viral genetic information by selectively influencing the relationship between viral RNA and the ribosomes [29, 30]. In cell systems where no virus synthesis takes place — e.g. SV-40 in 3T3 mouse cells — the activity of interferon apparently develops by a different mechanism, as a small amount is sufficient to block the virus-induced transformation of cells.

Examinations of the virus-stimulated endogenous interferon revealed a certain correlation between the virulence and cellular nucleic acid inhibitory action of certain virus mutants (e.g. poliovirus and VSV) and their interferon stimulating capacity [31, 32]. The cytopathic infective viruses are usually poor interferon inducers. For example, in the case of foot-and-mouth disease virus, activation of intracellular interferon resulted in intracellular persistence, a latent infection [33]. This fact seems to be of interest with regard to viral tumour genesis, as latent infection might promote the integration of viral and cellular information.

The capacity to stimulate endogenous interferon has been demonstrated with many oncogenic viruses [26, 27, 34, 35].

It has been shown that polyoma virus variants of different oncogenity for the mouse differ also in their capacity of inducing endogenous interferon [11, 12].

In the present experiments two variants of polyoma virus, differing in oncogenity for the hamster have been studied. Strain T-26, which is highly oncogenic for the hamster, stimulated interferon in embryonic hamster and mouse cell cultures more rapidly and in a higher titre than the considerably less oncogenic strain SE-55.

FRIEDMAN *et al.* [11] demonstrated that the mutant S of polyoma virus,

which induced a tumour in 80% of mice, was a weaker inducer of endogenous interferon both *in vivo* and *in vitro*, than the less oncogenic M mutant. When the highly oncogenic mutant S was injected simultaneously with the good interferon inducer, mutant M, there was a decrease in the number of tumours. It was therefore inferred that the different oncogenic properties of these mutants were related to the interferon stimulating capacity. The interferon induced by polyoma virus inhibits the development of virus particles, averting thus the virus-stimulated transformation of cells.

Inconsistent conclusions have been drawn by GOTLIEB-STEMATSKY *et al.* [12], who studied two other mutants of polyoma virus. They found that in embryonic mouse cell culture, the mutant $C^- H^+$, highly oncogenic for the mouse, stimulated interferon 4—12 times more than did the slightly oncogenic mutant $C^+ H^-$. Pronounced oncogenic activity was found to be associated also with other properties such as marked plaque formation, weak CPE and high sensitivity to interferon. The said authors believe that the marked interferon stimulating capacity of mutant C^-H^+ in embryonic mouse cell culture and its high sensitivity to interferon constitute the factor responsible for the resistance of the cells to the cytopathic effect of the virus, enabling it to remain viable in the host cell. These cirsumstances may provide favourable conditions for the integration of the virus and the cellular genomes, promoting thereby the transformation of cells.

Our results obtained with two polyoma virus variants in the embryonic hamster cell model agreed well with those obtained by GOTLIEB-STEMATSKY *et al.* in the mouse cell system, thus, oncogenity for the hamster and stimulation of interferon in the embryonic hamster cell culture, and, as the quoted authors, we, too, observed a relationship between the oncogenic activity *in vivo*, the interferon stimulating capacity *in vitro* and the cytopathic effect of the polyoma virus variants.

However, the existence of highly oncogenic, yet low interferon inducer Friedman and Rabson mutants of polyoma virus suggests that there is no interdependence between these two phenotypic markers. Nevertheless, the rate of the synthesis of infective virions and the development of the cytopathic effect are directly related to the rate of interferon synthesis and the amount of interferon produced. These facts by themselves do not provide a firm basis for the hypothesis that viral cell transformation was due to inhibition by interferon of the synthesis of infective virions. As already mentioned, numerous data are available concerning the suppression by exogenous interferon of the transforming and oncogenic effects of the viruses. Further experiments will have to clarify the precise role of interferon in viral tumour genesis. The enhancing or suppressive influence of interferon on the oncogenic property of the virus might depend also on the circumstances under which the interferon action develops.

The question whether or not there is preformed interferon in normal cells has been a matter of dispute. The available experimental data are insufficient to permit an equivocal conclusion.

The interferon induced by bacterial endotoxin *in vivo* does not differ from the virus-stimulated interferon except for its higher molecular weight [36, 37]. The endotoxin-stimulated interferon is characterized by its rapid appearance in the blood and resistance to inhibitors of protein synthesis [38]. This seems to suggest that the high molecular weight interferon is present in the organism in a preformed state and becomes activated by the invading endotoxin.

Many authors have studied the role of reticuloendothelial cells, leukocytes and macrophages in interferon formation [39—41]. These cells, particularly the macrophages, may be regarded as an important system of protection against viral infections [42, 43]. The spleen is known to play an important role in interferon synthesis [44]. In cultivated leukocytes interferon was stimulated very rapidly and in high titre by Newcastle Disease virus [45].

The present studies have revealed the presence of an inhibitor capable of suppressing the synthesis of Chikungunya virus in the nutrient medium of non-infected cells [46, 47]. A closer examination of the inhibitor's properties indicated its classification as an interferon-like substance. The action of the inhibitor could be suppressed with actinomycin D.

In later studies SMITH and WAGNER [48] have demonstrated the presence of an interferon-like inhibitor in non-infected rabbit macrophage cultures prepared by mobilization with intraperitoneally administered glycogen. According to our results non-infected peritoneal exudate cells produce interferon in higher titres if they are cultivated *in vitro*. The probability cannot be excluded that under such conditions the unusual environment may have a stimulatory influence on the spontaneous interferon production of the RES cells; in this case, however, the induction of interferon by latent virus, present either *in vivo* or *in vitro*, should always be taken into consideration.

Thus, the available evidence is insufficient to decide, whether or not the RES cells contain preformed interferon and, if they do, in what form; and to decide the precise role of that "normal" inhibitor in the organism's protection against viral infection.

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SEROLOGICAL AND PHAGE TYPING OF PSEUDOMONAS AERUGINOSA INVADING A MUNICIPAL WATER SUPPLY

By

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Summary. The source of a heavy pollution by *Ps. aeruginosa* in the Szeged water supply was detected by serological and phage typing. From 34.5% of peripheral drinking water specimens and from 11.7% of specimens taken from the incriminated water plant *Ps. aeruginosa* strains belonging to serogroup 4a, 4c and identical in phage sensitivity were isolated. After reconstruction of the plant *Ps. aeruginosa* disappeared from the water system.

In view of the outdated condition of the Szeged municipal distribution mains the bacteriological analysis of drinking water has been performed with increased attention for years. In October, 1967, a strikingly high degree of pollution with *Pseudomonas aeruginosa* became evident. In the Szeged water supply this organism had infrequently been detected. In the present paper we give an account of investigations into the source of the pollution.

Materials and methods

Drinking water specimens were taken twice weekly at 5 routine sampling sites in each of the three districts of the city. Control examination of the water plants was performed at least at monthly intervals. Special attention was devoted to Plant I established in 1904, as its out-of-date technical facilities presented a constant hazard of pollution.

Bacteriological examinations. As prescribed in the Hungarian standard for the bacteriological examination of water [1] 50 ml water were passed through Co 5 membrane filter. After placing the filter on Endo agar and incubating at 37°C for 24 and 48 hours, suspicious colonies were streaked onto nutrient agar and desoxycholate citrate agar plates. Identification of *Ps. aeruginosa* was performed as described in [2.]

Serological typing of *Ps. aeruginosa* was performed on the basis of LÁNYI's antigenic scheme and method [2].

Phage typing. Twenty standard phages obtained from the Central Public Health Laboratory, London, and 11 phages isolated from LÁNYI's *Ps. aeruginosa* type strains [2] were used. The phages were propagated on suitable type strains and were used in routine typing dilution.

Results

Between October 3 and November 8, 1967, a total of 133 drinking water specimens taken from peripheral sampling sites were examined. *Ps. aeruginosa* was cultured from 46 specimens (34.5%). During the same period of time 6 out of 51 specimens taken from Plant I were positive for this organism

(11.7%). The strains belonged uniformly to serogroup 4a, 4c and were identical in phage sensitivity. Laboratory examinations indicated that two underground basins of Plant I were polluted: the effluent water contained *Ps. aeruginosa* while the influent water supplying the basins by gravity flow from wells showed no evidence of contamination.

In the beginning of November, 1967, Plant I was disconnected from the municipal water system. After emptying the basins several minute cracks were disclosed on the concrete casing through which the ground water had gained access. The damage was supposed to be associated with the effect of blasting a wreck of bridge in the nearby river. The sanitary survey also revealed that until about 30 years ago the plant and the neighbouring ophthalmology clinic had been separated by a street in the axis of which ran a sewer. When in the thirties the street was cancelled the river-side outlet of the sewer was blocked up. The present investigations indicated that the long-forgotten sewer ran at a distance less than 5 metres from the two underground basins and its other end still communicated with the main sewer of the district. Because of its defective shell, by the conveyance of ground water, it polluted the reservoirs after the damage caused by blasting.

From November the municipal water system was supplied by Plants II, III and V situated in different districts of the city. These plants produced satisfactory water: all 75 specimens taken from them were negative. The overwhelming majority of peripheral samples were also negative; only some sampling sites yielded *Ps. aeruginosa* positive cultures (6 out of 374 specimens = 1.6%). From March till July, 1968, all samples were negative. In July *Ps. aeruginosa* was isolated from 2 peripheral specimens (serogroups 4a, 4d and 11).

In July the final stage of the reconstruction of Plant I began. During the 3-week period 45 specimens were taken from various sites in the plant and from the wells. The number of specimens positive for *Ps. aeruginosa* was 26 (57.7%). The high degree of pollution was attributed to various factors as rebuilding, pipe laying and earthwork. The isolated *Ps. aeruginosa* strains fell into various serogroups and phage types. In addition to 4a, 4c representing the sole serogroup, during the mass pollution strains belonging to serogroups 4a, 4d, 6 and 11 were encountered. Corresponding to the serogroup distribution, the strains differed in phage sensitivity.

Laboratory examination indicated that by July 26 the bacterial pollution of Plant I had been completely eliminated. The plant was then reconnected into the municipal water system.

The results of bacteriological examinations are summarized in Table I. It is interesting that in the drinking water specimens examined coliform bacteria occurred very infrequently. Not more than 17 coliform positive specimens were encountered (11 of them were taken in Plant I during reconstruction and 6 of them originated from one peripheral sampling site).

Table I

Incidence of Ps. aeruginosa serogroups and phage types in specimens taken from the Szeged water supply

Time of examination	Source of specimens	No. of specimens	No. of positive specimens	Polluted specimens, %	Serogroup and phage type distribution		
					No. of strains typed	Serogroup	Phage type
Oct. 3 — Nov. 8, 1967	Peripheral pipes	133	46	34.5	43	4a, 4c	7/21/24/119x/M_4/141/68/1214
	Plant I	51	6	11.7	4	4a, 4c	7/21/24/119x/M_4/141/68/1214
Nov. 9, 1967— March 1, 1968	Peripheral pipes	374	6	1.6	4	4a, 4c	7/21/24/119c/M_4/141/68/1214
	Plants II, III, V	75	—	—			
July 4— July 24, 1968	Peripheral pipes	102	4	3.9	2	4a, 4d	7/16/21/24/44/68/F_8/109/119x/1214/M_4/C_{11}
					2	11	21/68/M_4/C_{11}
	Plant I	45	26	57.7	4	4a, 4c	7/21/24/119x/M_4/141/68/1214
					2	4a, 4d	7/16/21/24/44/68/F_8/109/119x/1214/M_4/C_{11}
					4	6	7/21/68
					11	11	21/68/M_4/C_{11}

Discussion

Although the pollution of drinking water with *Ps. aeruginosa* is not uncommon, in the literature there are relatively few papers discussing the sanitary importance of this organism. The data indicate that *Ps. aeruginosa* appears in drinking water as a faecal contaminant [3—7]. Accordingly, its presence is undesirable and several water sanitation regulations, including the Hungarian national standard, regard such waters as unsafe for human consumption.

In the available literature there were no data for a mass invasion of a municipal water system by *Ps. aeruginosa*. This organism occurs in drinking water usually as a sporadic contaminant. As detected by the same technique as used in the present examinations, *Ps. aeruginosa* occurred in rural piped water specimens in 0.6% [6] and in the Budapest municipal water supply in 0.25% [7]. As seen in Table I, during the invasion of the Szeged water system the organism was recovered from 34.5% of the specimens.

It is interesting to compare the serogroup distribution of *Ps. aeruginosa* strains isolated from different water specimens. Seven out of 24 strains isolated from rural piped water samples by LÁNYI, GREGÁCS and ÁDÁM [6] and 6 out of 12 strains isolated from the Budapest water supply by NÉMEDI and LÁNYI [7] belonged to serogroup 4a, 4c responsible for the mass pollution in Szeged. Strains belonging to this serogroup occurred frequently not only in piped water, but also in wells, surface waters and sewage [6, 7]. These specimens, however, yielded also other serogroups, while in Szeged at the time of the mass pollution the isolates were uniform is serological properties and phage sensitivity. This finding indicates that the pollution originated from one single source.

Field survey and laboratory examinations indicated that the pollution of the Szeged water supply which caused fortunately no human infections, was due to a technical deficiency. It may thus be concluded that in addition to the routine checking of the operation of water plants one should always consider the potential danger of contamination arising from ground motion.

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PROPERTIES OF ACETOHYDROXYACID SYNTHETASE IN MYCOBACTERIUM PELLEGRINO

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Summary. Acetohydroxyacid synthetase extracted from *Mycobacterium pellegrino* was similar in properties and coenzyme requirement to the corresponding enzyme in other bacteria. The activity of the enzyme was inhibited by valine.

The enzyme was slowly inactivated by mild effects as dilution, alkalization over pH 8, substrate concentration over 15 *mM* and temperature over 33°C. Inactivation was preceded by a transient increase in activity and decrease in valine sensitivity. Valine exerted a protective action against the above effects.

The substrate saturation curve was sigmoid in type except a normal hyperbolic part in the range of 2—15 *mM* substrate concentration where Km corresponded to 1.61 *mM*. In the presence of valine the curve lost its sigmoid shape. Cofactors, in addition to valine, exerted a stabilizing effect.

Acetohydroxyacid synthetase is the first enzyme in the common pathway of the biosynthesis of branched-chain amino acids. The inhibitory effect of valine on this enzyme was first observed by Umbarger and Brown [1]. Later several authors described the valine sensitivity of this enzyme in various microorganisms [2—8]. The physiological effect of valine was shown in *Escherichia coli* K 12 [9] and in *Pseudomonas aeruginosa* [10]. In *Mycobacterium pellegrino* valine coordinatively increases the amount of enzymes involved in valine and isoleucine biosynthesis and exerts at the same time a transient inhibitory effect on growth [8]. As this effect may be explained by the valine sensitivity of the first common enzyme, acetohydroxyacid synthetase, we thought to be of interest to study the properties of this enzyme.

Materials and methods

Methods of cultivation of *M. pellegrino* was described in our previous papers [8, 11]. Enzyme extract was prepared from 24 hour cells derepressed by valine.

Isolation of the enzyme. The cells were washed twice in distilled water by centrifugation and resuspended at 0°C in the following solution: phosphate buffer (pH 7.8), 0.4 *M;* $MgCl_2$, 0.01 *M;* TPP, 100 μg/ml. Dry weight content of the suspension was 6—8 mg/ml. All further manipulations were carried out at 0°C.

The cells were disrupted by ultrasonic treatment 3 times for 3 minutes with an MSE Mullard ultrasonifier at 20 Kc and 100 W. The cellular debris was removed by centrifugation at 16,000 *g* for 20 minutes, then the crude extract was saturated to 38% with ammonium sulphate (pH 6.8) and after 1 hour standing the precipitate was removed by centrifugation at 16,000 *g* for 20 minutes. The supernatant was saturated to 68% with ammonium sulphate (pH 7.2) and after 2 hours standing the mixture was centrifuged. The precipitate was storable at 0°C for 2 days. Before use the precipitate was dissolved in the above phosphate buffer supplemented with 5 μM valine to 10 mg protein per ml concentration. The solution was then passed through Sephadex G-25 column equilibrated with the same buffer.

The reproducibility of the results was improved by the stabilizing effect of 5 μM valine. As the protein solution was diluted at least tenfold, its concentration exerted no effect on the reaction velocity.

The experiments were performed with the protein solution prepared in the above manner. Unless otherwise indicated, the specific activity of the preparation was higher by 4—6 times than the activity of the crude extract.

Assay of enzyme activity. The method of UMBARGER and BROWN was used [1]. The reaction mixture contained: phosphate buffer (pH 7.8), 0.1 *M;* $MgCl_2$, 0.01 *M;* thiamine pyrophosphate, 0.2 *mM;* flavinadenine dinucleotide, 0.01 *mM;* sodium pyruvate, 0.04 *M.* The enzyme reaction was carried out at 28°C. The α-acetolactic acid was determined after terminating the reaction with 10% sulphuric acid as described by WESTERFELD [12].

α-Aceto-α-hydroxybutyric acid was assayed by the microbiological method of LEAVITT and UMBARGER [13].

Specific activity was defined as the number of micromoles of acetohydroxyacid per hour per milligram of protein. Protein was measured by the method of LOWRY *et al.* [14].

Commercial analytic grade reagents were used.

Abbreviations. TPP = thiamine pyrophosphate; FAD = flavinadenine dinucleotide.

Results

Effect of cofactors on acetohydroxyacid synthetase activity. The activity of the cell-free extract and of the purified preparation depended considerably on the composition of the reaction mixture (Table I).

Table I

Acetohydroxyacid synthetase activity in various reaction mixtures

Complete reaction mixture	0.078 μM α-acetolactic acid
— TPP	0.008 μM α-acetolactic acid
— FAD	0.065 μM α-acetolactic acid
+ L-valine 0.1 *mM*	0.054 μM α-acetolactic acid
— Mg^{++}	0.062 μM α-acetolactic acid
0.01 *M* phosphate buffer	0.046 μM α-acetolactic acid
0.1 *M* tris buffer substituted for phosphate	0.048 μM α-acetolactic acid

Complete reaction mixture: phosphate buffer (pH 7.8), 0.1 *M;* $MgCl_2$, 0.01 *M;* TPP, 0.1 *mM;* FAD, 0.01 *mM;* pyruvic acid, 20 *mM;* protein, 0.25 mg/ml. Reaction time, 20 minutes at 28°C.

TPP is an essential cofactor of the enzyme. When Mg ions or FAD were omitted the activity decreased slightly. Removal of protein-bound TPP, Mg^{++} and FAD by gel filtration or dialysis caused the inactivation of the enzyme. During dialysis a transient increase in activity with decreased valine sensitivity was observed before inactivation.

The activity of the enzyme was inhibited by valine and the enzyme was stabilized by high concentrations of phosphate.

Effect of protein concentration. In the range of 0.4—2.0 mg protein per ml the reaction rate was proportional to the protein content of the system.

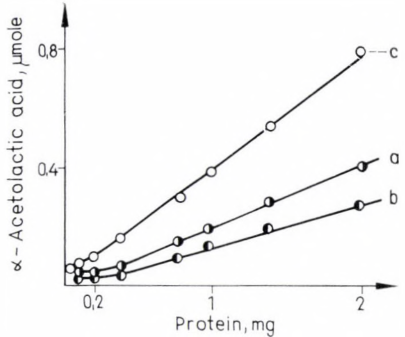

Fig. 1. Acetohydroxyacid synthetase activity versus protein concentration. Reaction mixture: phosphate buffer (pH 7.8); 0.1 *M*; $MgCl_2$, 0.01 *M*; TPP, 0.1 *mM*; FAD, 0.01 *mM*; pyruvic acid, 40 *mM*. Reaction time, 15 and 30 minutes at 28°C. a = acetolactic acid produced in 15 minutes; b = acetolactic acid produced in 15 minutes in the presence of 0.1 *mM* L-valine; c = acetolactic acid produced in 30 minutes

Under 0.4 mg protein per ml the activity decreased (Fig. 1); the decrease was not influenced by 0.1 *mM* TPP, 0.1 *mM* valine, 0.1 *mM* isoleucine or 0.01 *mM* FAD. This finding was probably due to an activation taking place during the reaction time. The reaction time could not be shortened because of the low protein concentration. This explanation is confirmed by the following observation. If the enzyme was gel filtered in the absence of valine the activity in some experiments increased and the amount of the final product was proportional to the protein concentration in the whole range of the experiment. The increase in activity was always accompanied by a decrease in valine sensitivity.

Effect of pH on reaction rate and valine sensitivity. The effect of pH on the reaction rate was examined in 0.1 *M* phosphate buffer. Maximum rate

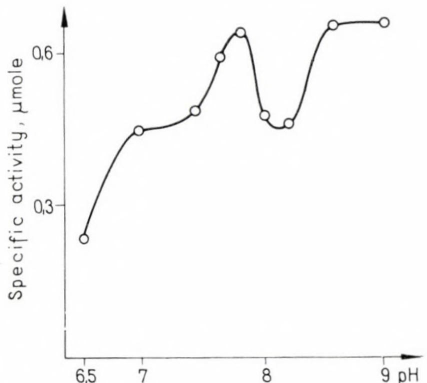

Fig. 2. Acetohydroxyacid synthetase activity versus pH. Reaction mixture: phosphate buffer, 0.1 *M*; $MgCl_2$, 0.01 *M*; TPP, 0.1 *mM*; FAD, 0.01 *mM*; pyruvic acid, 20 *mM*; protein, 0.25 mg/ml. Reaction time, 15 minutes at 28°C

was observed at pH 7.8. Over pH 8.0 there was a transient decrease, then a secondary increase (Fig. 2). The reaction rate was, however, proportional to time only up to pH 8.0, then a transitional activation was followed by a decreasing tendency in activity.

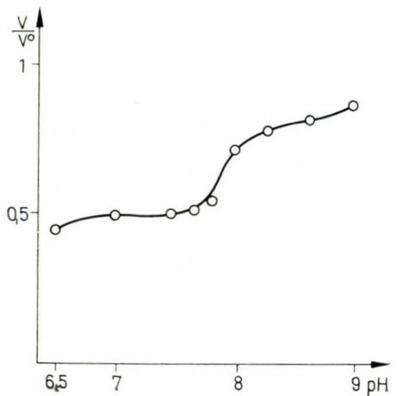

Fig. 3. Effect of pH on valine sensitivity of acetohydroxyacid synthetase. Reaction mixture: phosphate buffer, 0.1 *M;* $MgCl_2$, 0.01 *M;* TPP, 0.1 *mM;* FAD, 0.01 *mM;* pyruvic acid, 20 *mM;* protein 0.25 mg/ml. Reaction time, 15 minutes, at 28°C. Enzyme activity was inhibited with 1 *mM* L-valine

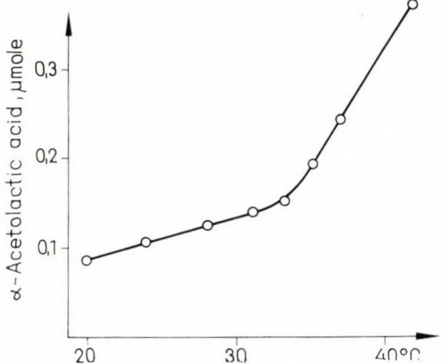

Fig. 4. Effect of temperature on acetohydroxyacid synthetase activity. Reaction mixture: phosphate buffer (pH 7.8), 0.1 *M;* $MgCl_2$, 0.01 *M;* TPP, 0.1 *mM;* FAD, 0.01 *mM;* pyruvic acid, 20 *mM;* protein, 0.65 mg/ml. Reaction time, 15 minutes

Valine sensitivity depended significantly on pH (Fig. 3). It remained almost unchanged up to pH 8.0, then decreased rapidly.

Effect of temperature on reaction rate and valine sensitivity. In the range of 20 – 30°C the reaction rate increased slowly; over 30°C there was a rapid rise (Fig. 4). Valine sensitivity somewhat increased between 20 and 30°C and decreased rapidly over 33°C (Fig. 5).

This finding indicates that over 30°C the enzyme suffers an important change in structure. This change has been confirmed by experiments on valine

sensitivity in the range of 20—42°C. Valine was added partly before, partly during reaction. At 20°C the inhibitory effect was the same when valine was added at 0, 10 or 20 minutes. In contrast, at 42°C addition of valine at 5 minutes exerted a weaker inhibition. At 10 minutes valine was almost ineffective. When the enzyme was preincubated without substrate for 10 minutes at

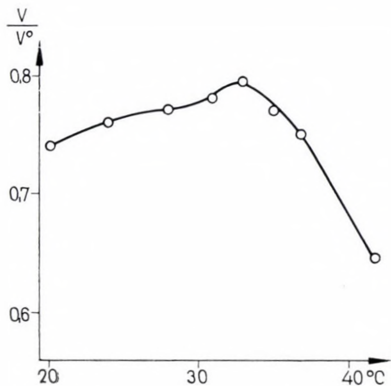

Fig. 5. Valine sensitivity of acetohydroxyacid synthetase activity versus reaction temperature. Reaction mixture: phosphate buffer (pH 7.8), 0.1 *M;* $MgCl_2$, 0.01 *M;* TPP, 0.1 *mM;* FAD, 0.01 *mM;* pyruvic acid, 20 *mM;* protein, 0.65 mg/ml. Reaction time, 15 minutes

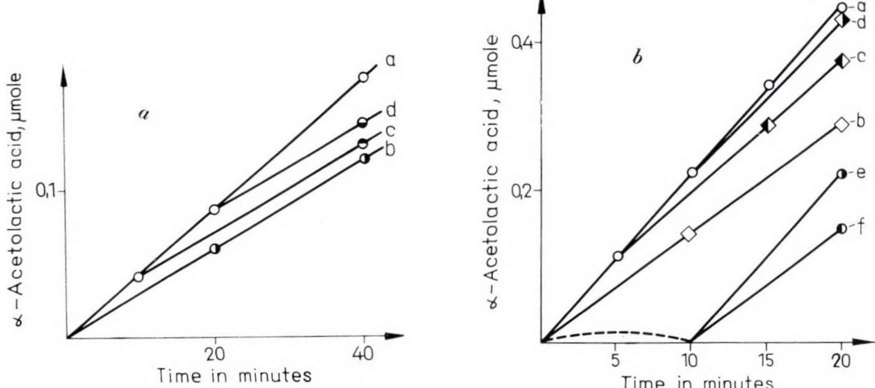

Fig. 6a. Changes in acetohydroxyacid synthetase activity as an effect of valine added during reaction at 20°C. Reaction mixture: phosphate buffer (pH 7.8), 0.1 *M;* $MgCl_2$, 0.01 *M;* TPP, 0.1 *mM;* FAD, 0.01 *mM;* pyruvic acid 20 *mM;* protein, 0.5 mg/ml. a = enzyme activity in the absence of valine; b = enzyme activity in the presence of 0.1 *mM* L-valine; c = changes in activity after adding 0.1 *mM* L-valine at 10 minutes; d = changes in activity after adding 0.1 *mM* L-valine at 20 minutes

Fig. 6b. Changes in acetohydroxyacid synthetase activity as an effect of valine added during reaction at 42°C. Reaction mixture: phosphate buffer (pH 7.8), 0.1 *M;* $MgCl_2$, 0.01 *M;* TPP, 0.1 *mM;* FAD, 0.01 *mM;* pyruvic acid, 20 *mM;* protein, 0.5 mg/ml. a = enzyme activity in the absence of valine; b = effect of 0.1 *mM* valine; c = effect of 0.1 *mM* valine added at 5 minutes; d = effect of 0.1 *mM* valine added at 10 minutes; e = reaction mixture preincubated without pyruvic acid at 42°C for 10 minutes; f = 0.1 *mM* valine and pyruvic acid was added to the preincubated reaction mixture at 10 minutes

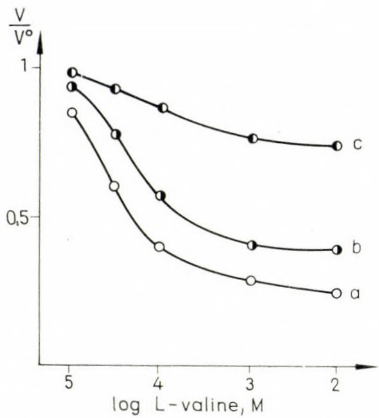

Fig. 7. Inhibition of acetohydroxyacid synthetase activity with valine. Reaction mixture: phosphate buffer (pH 7.8), 0.1 *M;* $MgCl_2$, 0.01 *M;* TPP, 0.1 *mM;* FAD, 0.01 *mM;* pyruvic acid, 2, 10 or 40 *mM;* protein, 0.63 mg/ml. Valine concentration varied between 10 μM and 10 *mM*. Reaction time, 15 minutes at 28°C. a = Enzyme activity in the presence of 2 *mM* pyruvic acid; b = 10 *mM* pyruvic acid; c = 40 *mM* pyruvic acid

42°C, valine sensitivity was the same as at 0 minute in the above experiment and the reaction rate remained unchanged (Figs 6a, 6b).

Effect of substrate concentration on valine sensitivity. The inhibitory effect of valine highly depends on the substrate concentration (Fig. 7). Valine sensitivity was the highest at low substrate concentration but no complete inhibition was observed even in the presence of 10 *mM* valine. Increasing of the substrate concentration suspended the inhibitory effect of valine (Fig. 8).

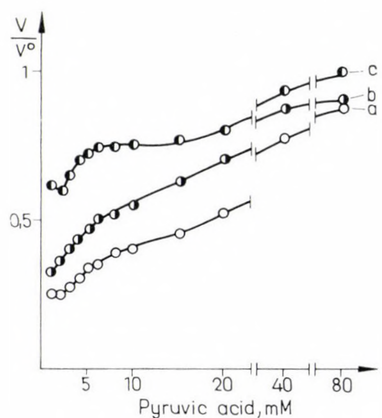

Fig. 8. Changes in valine sensitivity as an effect of substrate concentration. Reaction mixture: phosphate buffer (pH 7.8), 0.1 *M;* $MgCl_2$, 0.01 *M;* TPP, 0.1 *mM;* FAD, 0.01 *mM;* protein, 0.63 mg/ml. Pyruvic acid concentration varied between 1 and 80 *mM* at 10 *mM*, 100 μM and 50 μM L-valine concentration. Reaction time, 15 minutes at 28°C. a = Inhibitory effect in the presence of 10 *mM* L-valine; b = 100 μM L-valine; c = 50 μM L-valine

The inhibitory effect was highly specific for L-valine. While L-isoleucine inhibited slightly, D-valine was totally ineffective.

Effect of substrate concentration on the reaction rate. At pyruvic acid concentrations over 2 *mM* a strong activating effect was observed; at concentrations higher than 10 *mM* a definite inhibition occurred. The linear part of the reciprocal plot drawn according to LINEWEAVER and BURK fell in the range of 2—15 *mM* pyruvic acid concentration. The Km value obtained in this manner was 1.61 *mM* (Fig. 9). At pyruvic acid concentrations higher than

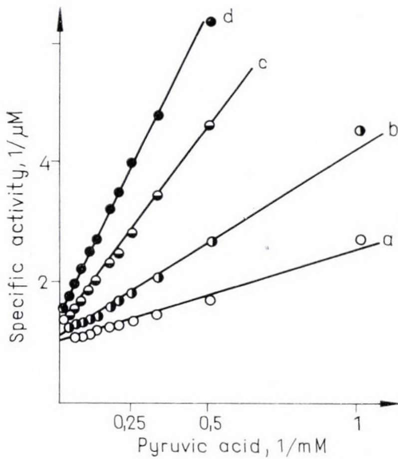

Fig. 9. Effect of pyruvic acid concentration on acetohydroxyacid synthetase activity in the presence of different valine concentrations. Reaction mixture: phosphate buffer, 0.1 *M*; $MgCl_2$, 0.01 *M*; TPP, 0.1 *mM*; FAD, 0.01 *mM*; protein, 0.25 mg/ml. Pyruvic acid concentration varied between 1 and 100 *mM*. a = Enzyme activity without inhibitory agent; b = in the presence of 50 *μM* L-valine; c = 100 *μM* L-valine; d = 1 *mM* L-valine

10 *mM* substrate inhibition was indicated by the ascending course following the straight part of the curve. Addition of valine decreased the inactivating effect of the substrate. In the presence of 0.1 *mM* valine neither of these effects was observed.

Effect of α-ketobutyric acid on α-acetohydroxyacid production. In agreement with data for other microorganisms under the experimental conditions α-ketobutyric acid considerably decreased the amount of acetohydroxy acid formed from pyruvic acid (Fig. 10). In the presence of equivalent pyruvic acid and α-ketobutyric acid concentrations valine precursor was produced in very slight amounts. If the α-ketobutyric acid : pyruvic acid ratio was altered in favour of pyruvic acid, the amount of valine precursor increased; however, the absolute amount of isoleucine precursor formed at the same time remained practically unchanged.

Experiments on the stability of the enzyme. The enzyme described in this paper, similarly to acetohydroxyacid synthetases in other bacteria, is very

sensitive. Its protein structure and activity are considerably altered on storage even at 0°C. In storage experiments shown in Table II the protein concentration was 10 mg/ml in 0.4 *M* phosphate buffer containing 0.01 *M* magnesium chloride.

Without thiamine pyrophosphate the activity decreased by 25% as soon as after 2 hours. After 24 hours only 20—25% activity was demonstrated.

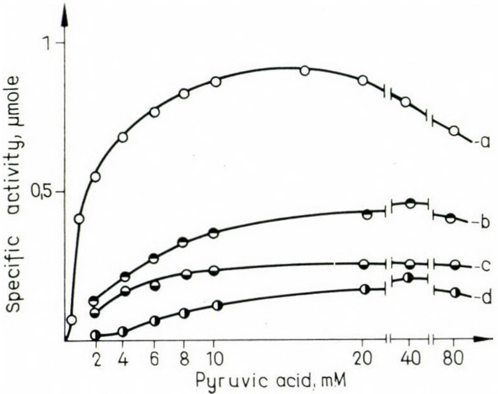

Fig. 10. Acetolactic acid and acetobutyric acid formation versus pyruvic acid concentration. Reaction micture: phosphate buffer (pH 7.8), 0.1 *M;* $MgCl_2$, 0.01 *M;* TPP, 0.1 *mM;* FAD. 0.01 *mM;* protein, 0.4 mg/ml. Pyruvic acid concentration varied between 1 and 80 *mM* in the presence or absence of 1 *mM* α-ketobutyric acid. Reaction time, 20 minutes at 28°C. a = amount of acetolactic acid formed in the presence of pyruvic acid (WESTERFELD's method); b = total amount of acetohydroxyacids formed in the presence of α-ketobutyric acid and pyruvic acid (WESTERFELD's method); c = amount of α-aceto-α-hydroxybutyric acid (agar diffusion assay); d = amount of α-acetolactic acid (calculated by subtracting the amount of α-aceto-α-hydroxybutyric acid from total acetohydroxyacid)

The decrease in activity was associated with a decrease in valine sensitivity. TPP or valine exerted a definite protective effect. In the presence of the latter only a 20—25% decrease in activity was observed after 24 hours.

Discussion

In properties and cofactor requirement acetohydroxyacid synthetase in *M. pellegrino* resembles the corresponding enzyme isolated from other microorganisms [2, 4—6]. The enzyme is highly sensitive to valine. The valine sensitive preparation is probably identical with the native enzyme. Its pH optimum is at 7.8. Under the experimental conditions of this study TPP exerted a stabilizing effect on the enzyme. FAD acted also as an activator but less definitely than in other organisms [2, 15, 16, 22]. In contrast to results obtained

for *Ps. aeruginosa* the enzyme could not be regenerated with FAD after heating [17]. Valine, a feedback inhibitor, protects the enzyme and decreases the rate of inactivation during storage. The stability of the enzyme is highly influenced by the concentration of the phosphate buffer and by the pH. The rate of "inactivation" increases under 0.1 *M* buffer concentration and over pH 8. Under these conditions first an increase in acitivity and a decrease in valine

Table II

Changes in the activity of protein solution stored at 0°C

Storage at 0°C	Specific activity	Valine sensitivity $\frac{V}{V^0}$
Zero time	0.82	0.55
2 hours	0.60	0.65
2 hours with 0.1 *mM* TPP	0.69	0.58
24 hours	0.19	0.90
24 hours with 0.1 *mM* TPP	0.58	0.70
24 hours with 0.1 *mM* TPP + 0.5 *mM* valine	0.65	0.65

Reaction mixture: phosphate buffer (pH 7.8), 0.1 *M*; $MgCl_2$, 0.01 *M*; TPP, 0.1 *mM* · FAD, 0.01 *mM*; pyruvic acid, 0.04 *M*; protein, 0.5 mg/ml. Valine sensitivity was determined in the presence of 0.1 *mM* valine.

sensitivity can be demonstrated. The changes in properties of enzyme over pH 8 are expressed by the characteristic double-peaked course of the pH curve (Fig. 2).

After several unsuccessful attempts we abandoned the aim to prepare a pure enzyme. Ammonium sulphate precipitation was used rather to obtain an active preparation with high protein concentration.

Ultrasonic treatment releases the enzyme from the milieu of the intracellular structure. Certain biosynthetic enzymes might be localized in a loose structural complex in the intracellular space. This hypothesis is supported by the findings of Wagner *et al.* [18—20]. After disintegration of the cell wall the deterioration of the enzyme complex begins. This process can be influenced by heating and alteration of pH and ionic strength. Valine molecules might play an important role in stabilization of the complex and, therefore, influence definitely the activity of the enzyme. The stabilizing and activity-influencing effect of valine could not be perfectly separated. The increase of concentration of valine makes the enzyme more compact and at the same time retards probably the loosening of the enzyme complex. At higher temperatures the effect of valine becomes less definite and, accordingly, higher concentrations are needed to ensure the stable conditions for the enzyme and the enzyme complex.

The loosening of the enzyme complex is accompanied by a structural change in the enzyme and, consequently, by an increase in activity as demonstrated in heating experiments. At higher temperatures, over 30°C, simultaneously with the decrease in valine sensitivity, as demonstrated by VARGA and HORVÁTH [15] in *Ps. aeruginosa*, the activity of the enzyme increases highly. Degradation of enzyme molecules insensitive to valine may cause a percentage increase in valine sensitivity. In our opinion the degradation of the enzyme begins with losing valine sensitivity. During storage of fresh enzyme solutions sometimes an increase in valine sensitivity and a parallel decrease in activity were shown.

It has been demonstrated that the presence of substrate increases the rate of inactivation. This finding explains the phenomenon of substrate inhibition. Exposure to 42°C decreased the valine sensitivity of the enzyme in the presence of the substrate molecule. This finding may be explained by the commencing degradation of the enzyme. Exposure to 42°C for 10 minutes (Fig. 6b) decreased very slightly the valine sensitivity; under the same conditions in the presence of pyruvic acid valine exerted practically no inhibitory effect.

On the basis of results obtained in *Ps. aeruginosa* by VARGA and HORVÁTH [21, 22] it occurred to us that, due to its small molecular size, the valine resistant enzyme in *M. pellegrino* might be separated by gel filtration, but the active fraction was excluded by Sephadex G-200. It may be supposed that the valine resistant fraction probably smaller in molecular size, was completely inactivated by gel filtration.

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INDUCTION AND MULTIPLICATION OF λ-PHAGE

IV. THE EFFECT OF PANFURAN

By

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Summary. Panfuran, 3-amino-6(2[5-nitro-2-furyl-vinyl])1,2,4-triazine HCl, a DNA synthesis-inhibiting agent, induces at 0.03 μg/ml concentration *Escherichia coli* strain K12 (λ-28) by similar kinetics as mitomycin-C. Mitomycin, panfuran and hydroxyurea resistant variants show no cross-resistance in respect to phage induction.

Kato *et al.* [1] described that panfuran, a nitrofuran derivative [3-amino-6[2(5-nitro-2-furyl-vinyl)]1,2,4-triazine HCl] induced at 0.1 μg/ml concentration a lysogenic *Escherichia coli* strain. They have assumed that panfuran (NFT) exerts no direct action on DNA but acts, corresponding to the theory of Goldthwait and Jacob [2] through DNA metabolites inactivating phage repressors.

In our first paper we demonstrated that mitomycin-C and hydroxyurea (HU) acted upon *E. coli* K12 (λ-28) by different kinetics. We have assumed that mitomycin-C induces the release of prophage by a more direct mechanism than hydroxyurea for which the Goldthwait—Jacob theory is well applicable [3, 4]. It seemed desirable to us to examine whether panfuran exerts a mitomycin or a hydroxyurea-type induction.

Woody-Carrer and Greenberg [5] described that part of nitrofurazone resistant *E. coli* S variants showed cross-resistance to certain radiomimetic substances including mitomycin-C. In view of these data and in order to explain the results of kinetic experiments we examined the inducibility and cross-resistance of different *E. coli* K12 (λ-28) variants resistant to mitomycin-C, hydroxyurea, panfuran or to bacteriostatic nitrofurans not exerting phage induction.

Materials and methods

Organisms. E. coli K12 (λ-28 ind^{+}) and *E. coli* K12 (λ cI-857 ind^{-})TL^{-} were used in induction experiments. *E. coli* C600 served as an indicator culture.

Resistant variants of E. coli K12 (λ-28). By tube dilution test the bacteriostatic concentrations of mitomycin, hydroxyurea, panfuran, N-(5-nitrofurfurylidene)-amino-guanidine HCl and N-(5-nitrofurfurylidene)-1-amino-hydantoin sodium (Table I) were determined. The cultures were trained to resistance by passaging them in the presence of gradually increasing concentrations of the agents. After 4 to 5 passages the resistant variants were isolated by streaking the cultures on agar plates. The resistance of the variants is shown in Table II.

The data indicate maximal concentrations allowing growth in broth incubated at 37°C for 24 hours.

Culture media. Minimal medium: DAVIS and MINGIOLI's [6] medium was supplemented with 0.00001% casein hydrolysate. Complete medium: broth or minimal medium was supplemented with 0.25% casein hydrolysate.

Experimental conditions were essentially the same as described in our previous paper. A 16-hour broth culture was transferred to the same medium (1 : 9) and incubated further for 2 hours. Then the culture was washed twice in distilled water and inoculated for induction experiments at 10^6 cells per ml. For shift down experiments inoculation was performed into minimal medium. Incubation was carried out for K12 (λ-28) at 37°C, for heat-inducible cultures at 28°C. The inductive substances were added to the medium at zero time. Heat induction was performed at 45°C for 15 minutes. After incubation viable counts and infective centre counts for the untreated culture and the concentration of complete phages in chloroform-treated cultures were determined at 28°C and at 37°C, respectively.

Results

Fig. 1 shows the effect of strain K12 (λ-28) of NFT at 0.03 μg/ml concentration found optimal in preliminary experiments. The production of complete phages was inhibited only over 1 μg/ml. In broth (Fig. 1a) the increase in infective centre counts started immediately. The concentration of induced complete phages began to increase over the spontaneous level only after a 40—45 minute lag. In the presence of chloramphenicol (CM) the number of infective centres increased but slightly and transiently and the production of complete phages was totally inhibited. The viable count decreased only

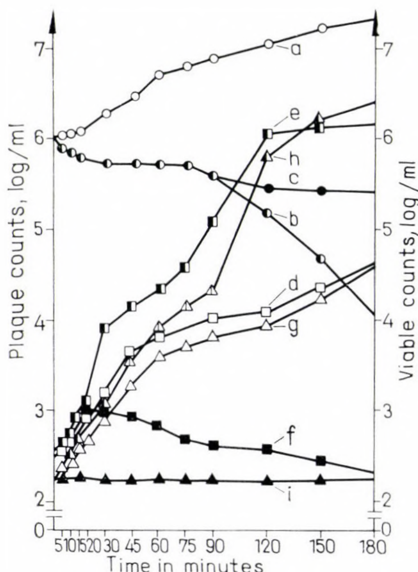

Fig. 1a. Inductive effect of NFT and influence of CM on induction in broth. Viable counts in not induced culture (a), with 0.03 μg NFT/ml (b), with 0.03 μg NFT + 20 μg CM/ml (c). Infective centre count in not induced culture (d), with 0.03 μg NFT/ml (e), with 0.03 μg NFT + 20 μg CM/ml (f). Number of free phages in not induced culture (g), with 0.03 μg NFT/ml (h), with 0.03 μg NFT + 20 μg CM/ml (i)

in cultures incubated without CM, where practically all cells became induced. In shift down state (Fig. 1b) there was also an increase in infective centre count; however, this change was not inhibited by CM. In experiments with mitomycin, CM exerted a very similar effect [7]. Complete phage production started only after a prolonged lag phase and the decrease in viable cell count was less than in broth. If minimal medium supplemented with 0.25% casein hydrolysate was used, the course of the process occupied an intermediary position betweenin duction types observed in the two above experiments.

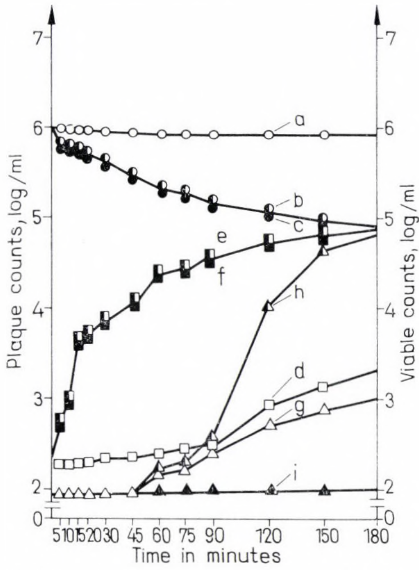

Fig. 1b. Inductive effect of NFT and influence of CM on induction in shift down state. Viable counts in not induced culture (a), with 0.03 μg NFT/ml (b), with 0.03 μg NFT + 20 μg CM/ml (c). Infective centre count in not induced culture (d), with 0.03 μg NFT/ml (e), with 0.03 μg NFT + 20 μg CM/ml (f). Number of free phages in not induced culture (g), with 0.03 μg NFT/ml (h), with 0.03 μg NFT + 20 μg CM/ml (i)

NFT at 28°C did not exert an inducing effect on *E. coli* K12 (cI-857). Heat induction and phage production at 37°C thereafter were not influenced by the concentration of the agent.

Fig. 2 demonstrates shift down experiments with *E. coli* K12 (λ-28) for mitomycin-C, hydroxyurea and NFT. It is seen that while as an effect of NFT and mitomycin-C the number of infective centres increased similarly, in the presence of hydroxyurea the increase started only after a considerable lag phase.

Mitomycin at 2°C brought about a small degree of induction. NFT at 2°C caused a 4- to 5-fold increase in infective centre count.

Fig. 3 presents data for resistant cultures as related to viable counts in the corresponding inoculum. The NFT resistant variant retained its induc-

ibility for mitomycin and hydroxyurea and showed a slow, but significant increase in infective centre counts (Fig. 3a). The mitomycin resistant variant remained completely inducible by NFT and less markedly by hydroxyurea (Fig. 3b). The hydroxyurea resistant culture could be induced by both NFT and mitomycin (Fig. 3c). In the latter organism the effect of mitomycin and panfuran was very similar to that in the parent strain.

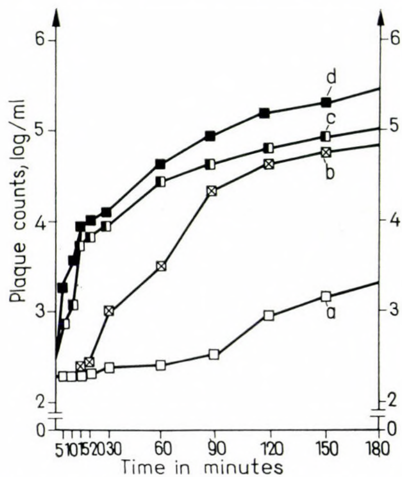

Fig. 2. Inductive effect of NFT, mitomycin-C and HU in shift down state. Infective centre counts in not induced culture (a), with 0.1 *M* HU (b), with 0.03 μg NFT/ml (c), with 1.0 μg mitomycin-C/ml (d)

In experiments with resistant cultures there was a slow rise in viable counts (2- to 3-fold increase in 180 minutes), that is, the metabolism of the cells was slower. The generation times for the cultures are presented in Table II.

It is seen that, in contrast to the finding of LEVIN and BORTHWICK [8] who showed that streptonigrin resistant *E. coli* K12(S) was inducible by streptonigrin, our strains resistant to NFT, mitomycin or HU were not induced by the corresponding substance. In order to approach more closely the mode of action of NFT, the inducing effect of this agent on *E. coli* K12 (λ-28) variants trained to various nitrofurans was examined.

Table I shows nitrofuran derivatives examined for phage induction. Infective centre counts were determined in broth at 37°C with strain K12 (λ-28).

Fig. 4 demonstrates the result of experiments on cross-resistance between furan derivatives exerting positive and negative effect. A variant of K12 (λ-28) trained resistant to 100 μg NF_3 and NF_4 per ml remained inducible with NFT.

Table II shows the cross-resistance of the variants to various agents and the generation times. It is evident that NFT resistance is accompanied

Table I

Nitrofuran derivatives examined in phage induction experiments

	Structure	Name
NF_3	O_2N–(furan ring, O)–CH=N–NH–C(=NH)–NH_2 · HCl	N-(5-nitro-furfurylidene)-amino-guanidine-hydrochloride
NF_4	O_2N–(furan ring, O)–CH=N–N–(ring: =S, NH, ONa)	N-(5-nitro-furfurylidene)-1-aminohydantoin sodium
NFT	O_2N–(furan ring, O)–CH=CH–(ring: =N, N–N)–NH_2 · HCl	3-amino-6[2(5-nitro-2-furyl-vinyl)] 1,2,4-triazine hydrochloride

by a considerable resistance to mitomycin. In contrast, the NFT resistance of the mitomycin resistant mutant increased but slightly.

In induction experiments an important factor is the first three hours of growth. During this interval the mitomycin sensitivity of the NFT resistant mutant was higher than that shown in Table II.

Table II

Cross-resistance and generation time of resistant variants

Variant	NFT µg/ml	Mitomycin µg/ml	Hydroxy-urea *M*	NF_3 µg/ml	NF_4 µg/ml	Generation time, minutes
K12 (λ-28)	1.5	2.5	0.125	8	16	24
K12 (λ-28)/mitomycin	3.1	20.0	0.125	8	16	38
K12 (λ-28)/NFT	100	15.0	0.09	16	62.5	54
K12 (λ-28)/HU	1.5	3.5	0.175	8	16	42
K12 (λ-28)/NF_3	6.2	5	—	75	31	32.5
K12 (λ-28)/NF_4	12.5	5	—	16	100	37

The data indicate concentrations allowing growth in broth after incubation at 37°C for 24 hours.

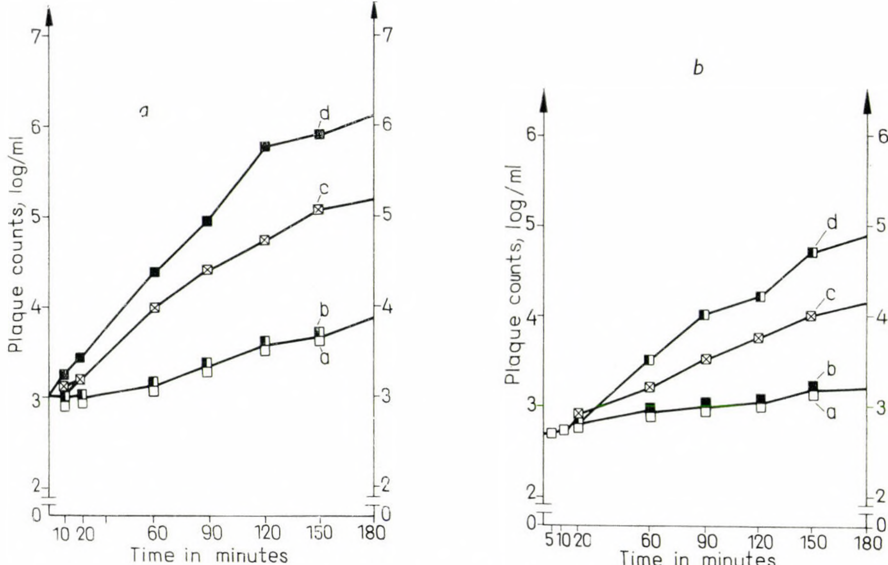

Fig. 3a. Inducibility of NFT resistant cells by HU and mitomycin-C in broth. Infective centre counts in not induced culture (a), with 0.03 μg NFT/ml (b), with 0.1 *M* HU (c), with 1 μg mitomycin-C/ml (d)

Fig. 3b. Inducibility of mitomycin-C resistant cells by HU and NFT in broth. Infective centre counts in not induced culture (a), with 1.0 μg mitomycin-C/ml (b), with 0.1 *M* HU (c), with 0.03 μg NFT/ml (d)

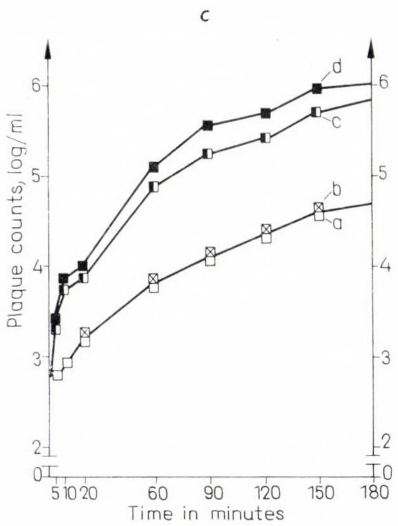

Fig. 3c. Inducibility of HU resistant cells by mitomycin-C and NFT in broth. Infective centre counts in not induced culture (a), with 0.1 *M* HU (b), with 0.03 μg NFT/ml (c), with 1.0 μg mitomycin-C/ml (d). The values of Figs 3a–c are calculated by dividing the plaque counts with the ratio of cell counts at t/t_0 times

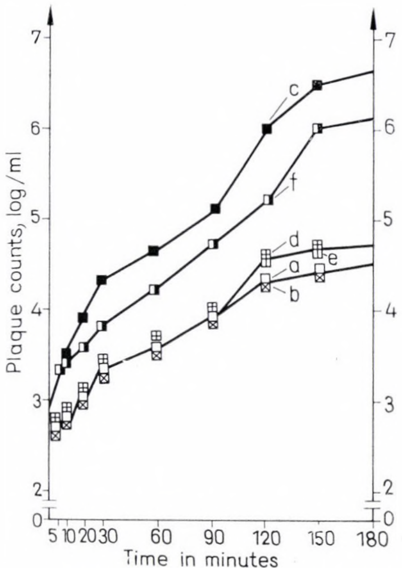

Fig. 4. Inducibility by NFT in broth of cells resistant to nitrofuran derivatives NF_3 and NF_4. Infective centre counts for NF_3 resistant cells in not induced culture (a), with 0.1 μg NF_3/ml (b), with 0.03 μg NFT /ml (c). Infective centre counts for NF_4 resistant cells in not induced culture (d), with 0.1 μg NF_4/ml (e), with 0.03 μg NFT/ml (f)

Discussion

Our experiments have shown that the inductivity of NFT, similarly to that of mitomycin and hydroxyurea, is associated with the ind$^+$ character: the substance was ineffective on the heat-inducible ind$^-$ variant. There was a similarity in every respect between the inductive effect of NFT and mitomycin-C [7]. In contrast, hydroxyurea exerted a different kind of inducing effect.

Although the experiments have not elucidated the mode of action of NFT for certain, it seems improbable that a Goldthwait—Jacob-type mechanism is involved. From the rapid increase in infective centre counts and the slight induction observed on cooling, it might be assumed that a disorder in DNA synthesis or a degradation of DNA is directly responsible for the release of prophage. This hypothesis has not been confirmed by cross-resistance experiments as there was no cross-resistance between mitomycin and NFT in respect to phage induction. This negative result does not disprove our hypothesis, it only indicates that the site of action of the two substances on DNA is different. In experiments with HU the NFT resistant strain gave less marked result; this finding may be due to the fact that, because of the decreased metabolic activity in the resistant variant, the hypothetical inductive DNA metabolites failed to reach the concentration needed.

Examination of inductive and not inductive nitrofuran derivatives indicated that *E. coli* K12 (λ-28) cells trained to resistance against non-inductive substances showed an unchanged inductibility (Fig. 4): the curves representing infective centre counts were similar in course for the parent and for the resistant cultures. Thus it may be concluded that growth-inhibiting and inducing effects in case of nitrofuran derivatives are localized at different sites of DNA. It may be assumed that the inductive effect of NFT is associated with the marked electron-acceptor character of the molecule, that is, with the conjugated double bond system of the agent.

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AMINO ACID UTILIZATION BY SEROLOGICALLY GROUPED PSEUDOMONAS AERUGINOSA STRAINS

By

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Summary. Amino acid utilizaton in defined medium by 233 *Ps. aeruginosa* strains belonging to 23 different serological groups has been examined. The strains had been isolated from a wide variety of sources and were selected so as to represent the average incidence of serological units.

The amino acid spectrum of *Ps. aeruginosa* is characterized as follows: L-alanine 99.6, gamma-amino-butyric acid 99.6, L-arginine 99.6, DL-aspartic acid 99.6, DL-glutamic acid 99.6, glycine 99.6, L-histidine 99.6, hydroxy-L-proline 99.6, DL-isoleucine 98.3, DL-leucine 98.3, L-lysine 99.6, DL-ornithine 99.6, DL-phenylalanine 99.6, L-proline 99.6, L-tryptophan 97.4, L-tyrosine 99.6, DL-valine 99.6% positive; L-cysteine, L-cystine, DL-methionine 100% negative. Different reactions varying even within individual strains were recorded in DL-norleucine, DL-norvaline, DL-serine and L-threonine.

It has been concluded that, in respect to amino acid utilization, *Ps. aeruginosa* is a homogeneous species.

Recent observations indicate that growth in defined medium with amino acids and other nitrogenous substances may be useful for the differentiation of aerobic pseudomonads [3, 8]. The present paper gives an account of the amino acid spectrum of serologically defined *Pseudomonas aeruginosa* strains isolated from a variety of sources.

Materials and methods

Strains. A total of 233 *Ps. aeruginosa* strains selected for a previous study on biochemical reactions [5] were used. The strains represented 23 different serological groups.

Basal medium. K_2HPO_4, 5 g; $MgSO_4$, 0.5 g; distilled water, 1000 ml.

Amino acid medium. To 1000 ml basal medium 1 g L-amino acid or 2 g DL-amino acid were added, the pH was adjusted to 7.0 and the solution was Seitz-filtered. The medium was then distributed aseptically at 2—3 ml portions into 100×11 mm tubes. The following amino acids were used: L-alanine (NBC), gamma-aminobutyric acid (Reanal), L-arginine (NBC), DL-aspartic acid (NBC), L-cysteine HCl (NBC), L-cystine (NBC), DL-glutamic acid (NBC), glycine (BDH), L-histidine (NBC), hydroxy-L-proline (NBC), DL-isoleucine (NBC), DL-leucine (NBC), L-lysine HCl (NBC), DL-methionine (NBC), DL-norleucine (Reanal), DL-norvaline (Reanal), DL-ornithine (Reanal), DL-phenylalanine (BDH), L-proline (NBC), DL-serine (NBC and Reanal), L-threonine (NBC), L-tryptophan (Reanal), L-tyrosine (NBC), DL-valine (NBC).

Inoculation and incubation of media and reading of results. Seeding was performed from agar slants incubated at 37°C overnight, then at room temperature for 3 days by immersing into the amino acid media a small amount of culture with a straight wire. The tubes were incubated at 37°C for 14 days. Tubes showing definite turbidity were recorded as positive.

Results

Table I shows the utilization of amino acids as sole sources of carbon and nitrogen. In preliminary experiments amino acid utilization in defined ammonium medium was also examined. As essentially the same results had been obtained in the absence and in the presence of ammonium salt, in the main experiments utilization was examined in defined medium containing in addition to the amino acid only potassium, magnesium, phosphate and sulphate ions.

From Table I it is evident that the strains behaved uniformly in amino acid spectrum. Rapid and abundant growth was recorded in L-alanine, gamma-aminobutyric acid, L-arginine, L-glutamic acid, L-histidine, hydroxy-L-proline, DL-ornithine, L-proline, L-tryptophan, L-tyrosine and DL-valine. Somewhat weaker and often late multiplication was observed in DL-aspartic acid, glycine, DL-isoleucine, DL-leucine, L-lysine and DL-phenylalanine. None of the isolates utilized L-cysteine, L-cystine and DL-methionine.

In DL-norvaline, DL-serine and DL-threonine part of the strains failed to grow in 14 days, part of them showed late utilization. These amino acid tests were repeated. In Table I it is seen that results obtained for three series of experiments differ not only in the beginning of growth but also in the number of positive and negative reactions. As to individual strains, the following results were obtained. In DL-norvaline 68 strains were uniformly positive and 55 strains were uniformly negative in the three series of experiments; 110 strains gave variable results. In DL-serine 83 strains showed identical (28 positive and 55 negative), 150 strains discrepant results. In DL-threonine 58 uniformly positive, 101 uniformly negative and 74 variable results were recorded. Growth in DL-norleucine was even less definite. In two serial tests 10 uniformly positive, 204 uniformly negative and 19 variable results were obtained.

Experiments performed with several strains in series of 10 tubes containing the above amino acids confirmed that the reactions were variable within one experiment and within the same strain.

Some strains showing late or irregular growth were tested in the corresponding amino acid by using washed bacteria as inoculum. In multiple tubes seeded with 10^2 and with 10^4 viable cells washed three times in distilled water the results were similar to those obtained by using agar slant inocula, only the time before visible growth developed was more uniform. The growth could be transferred serially in the same defined medium; turbidity developed frequently earlier in the subcultures than in the primary cultures.

Atypical strains are listed in Table II. One isolate (171005) utilized none of the amino acids tested. This strain had been shown to differ from other cultures in failing to utilize ammonium salt and urea as sole source of

Table I

Amino acid utilization by Ps. aeruginosa in defined medium
Number of strains, 233

	Growth in defined medium			
	1–2 days	3–6 days	7–14 days	negative
L-Alanine	232	—	—	1
Gamma-aminobutyric acid	232	—	—	1
L-Arginine	229	3	—	1
DL-Aspartic acid	199	32	1	1
L-Cysteine	—	—	—	233
L-Cystine	—	—	—	233
DL-Glutamic acid	232	—	—	1
Glycine	209	18	5	1
L-Histidine	229	3	—	1
Hydroxy-L-proline	231	1	—	1
DL-Isoleucine	178	50	1	4
DL-Leucine	80	148	1	4
L-Lysine	185	46	1	1
DL-Methionine	—	—	—	233
DL-Norleucine Exp. I	—	2	20	211
DL-Norleucine Exp. II	—	2	9	222
DL-Norvaline Exp. I	—	12	154	67
DL-Norvaline Exp. II	—	3	92	138
DL-Norvaline Exp. III	—	3	90	140
DL-Ornithine	232	—	—	1
DL-Phenylalanine	144	53	35	1
L-Proline	231	1	—	1
DL-Serine Exp. I	—	16	21	196
DL-Serine Exp. II	2	57	62	112
DL-Serine Exp. III	24	88	69	52
L-Threonine Exp. I	4	26	66	137
L-Threonine Exp. II	—	57	39	137
L-Threonine Exp. III	—	47	41	145
L-Tryptophan	220	6	1	6
L-Tyrosine	232	—	—	1
DL-Valine	232	—	—	1

nitrogen [5]. In media containing complex nitrogenous substances it did not differ in biochemical and cultural characters from typical *Ps. aeruginosa* strains. Among strains atypical in amino acid spectrum isolate 171453 was

Table II

Atypical strains

Designation	O antigen*	Atypical property
171 005	1	Fails to utilize amino acids in defined medium
171 103	3a, 3b	Fails to utilize DL-isoleucine and DL-leucine
171 161	3a, 3d	Fails to utilize DL-isoleucine and DL-leucine
171 258	4a, 4c	Fails to utilize L-tryptophan
171 259	4a, 4c	Fails to utilize L-tryptophan
171 314	5a, 5b, 5d	Fails to utilize L-tryptophan
171 453	9	Fails to utilize DL-isoleucine, DL-leucine and L-tryptophan
171 503	11	Fails to utilize L-tryptophan
171 506	11	Fails to utilize L-tryptophan

* LÁNYI's antigenic scheme [4].

also atypical in sugar breakdown [5]; the remaining cultures behaved in respect to other biochemical reactions as typical *Ps. aeruginosa* strains.

The present studies have shown that there is no association between amino acid utilization and serological properties. Strains characterized by early or by late amino acid utilization were more or less evenly distributed in all serogroups.

Discussion

There are few published observations on the amino acid reactions of *Ps. aeruginosa.* Some authors tested *Ps. aeruginosa* strains with amino acid reactions elaborated for the differentiation of *Enterobacteriaceae.* The findings of SHERRIS, SHOESMITH, PARKER and BRECKON [7], BÜHLMANN, VISCHER and BRUHIN [1], LYSENKO [6], HUGH and LEIFSON [2], ZIERDT and SCHMIDT [10] and LÁNYI [5] are in agreement that *Ps. aeruginosa* produces arginine dihydrolase. The lysine decarboxylase reaction is negative according to LYSENKO [6], VÉRON [9], HUGH and LEIFSON [2] and LÁNYI [5]. The ornithine decarboxylase reaction was also described as negative by LYSENKO [6], HUGH and LEIFSON [2] and LÁNYI [5]. *Ps. aeruginosa* produces no tryptophan deaminase [5].

There are less data for amino acid utilization. JESSEN [3] examined the amino acid spectrum of 51 fluorescent pseudomonad cultures. *Ps. aeruginosa* utilized glutamic acid, ornithine, alanine, glycine and arginine rapidly,

gave variable reactions in serine and leucine and grew weakly or slowly in cystine. The present studies, with the exception of growth in cystine, confirmed JESSEN's results. JESSEN's table indicates that some amino acids may be useful for the differentiation of *Ps. aeruginosa* from other pseudomonads.

Amino acid utilization in defined medium was extensively studied by STANIER, PALLERONI and DOUDOROFF [8]. They examined a total of 267 aerobic pseudomonads including 29 strains of *Ps. aeruginosa*. They showed that alanine, gamma-aminobutyric acid, arginine, aspartic acid, glutamic acid and ornithine were utilized by all *Ps. aeruginosa* strains. These data are in agreement with the present finding.

According to the results of STANIER *et al.* serine was utilized by 4, leucine by 28, isoleucine by 15, valine by 25, lysine by 26, tyrosine by 28, phenylalanine by 7, tryptophan by 21 out of the 29 *Ps. aeruginosa* strains examined. In the present study the above amino acids, with the exception of serine, were utilized by almost all *Ps. aeruginosa* strains. The reason for the disagreement is obviously due to a difference in methods. STANIER *et al.* used plated media seeded by replica method and incubated for 4 days. This technique is evidently less sensitive than examination in fluid medium incubated for 14 days. If early readings of fluid media are considered there is no essential difference between the findings.

The results indicate that *Ps. aeruginosa* is a homogeneous species not only in biochemical characters used widely for defining bacteria [5] but also in amino acid spectrum. The present study may be regarded as a further step in evaluating the suitability of amino acid tests for the classification of pseudomonads.

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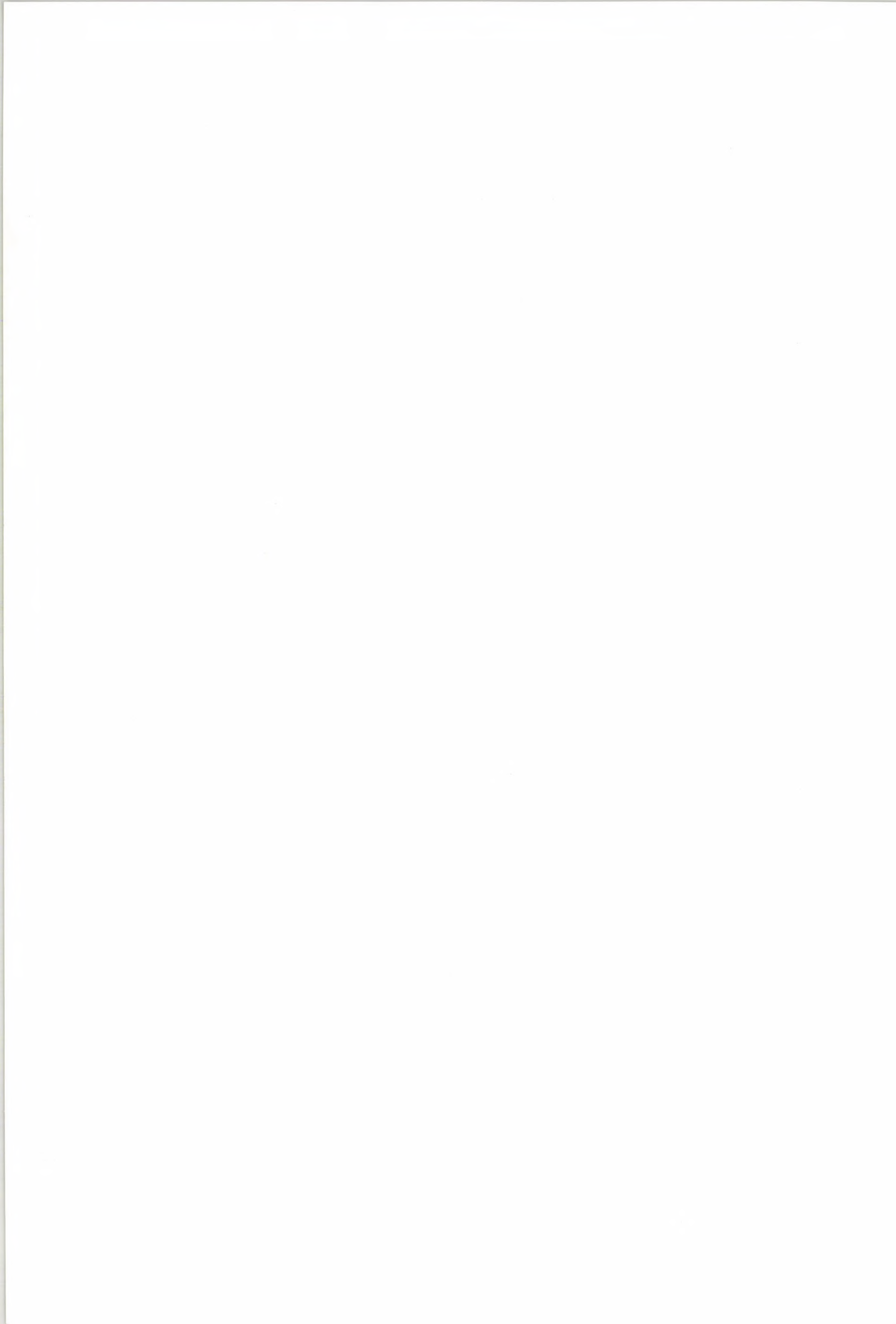

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THE EFFECT OF HYPOTHERMIA ON ALLERGIC-TYPE SKIN REACTIONS

By

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Summary. Passive cutaneous anaphylaxis and dinitrochlorobenzene hypersensitivity in mice and inverse passive Arthus phenomenon in guinea pigs have been studied. When the reactions were elicited in hypothermic animals the symptoms either failed to develop or were significantly milder than in normothermic control animals. The pathomechanism of these skin reactions has been discussed.

In previous experiments we have shown that both active and passive anaphylactic shock in the guinea pig can be prevented by cooling the animals [1, 2]. We have also demonstrated that hypothermia highly inhibits the anaphylactoid reaction in rats induced by dextran or ovalbumin [3]. The present paper gives an account of the effect of cooling on allergic type skin reactions.

Materials and methods

Passive cutaneous anaphylaxis was studied in *mice*. Antiovalbumin rabbit serum (5 μg N) was injected into the shaven abdominal skin and after a 2 hour interval the homologous antigen (1.5 mg ovalbumin mixed with an equal part of Evans blue) was injected intravenously The result was read 45 minutes after the eliciting injection at transmitted light by considering the diameter of the blue coloration of the skin: $\pm = 1-3$ mm; $+ = 3-5$ mm; $++ = 5-10$ mm; $+++ = >10$ mm.

Dinitrochlorobenzene (DNCB, Reanal) hypersensitivity was induced as follows. The agent was dissolved freshly in acetone, then 50 μg DNCB in 0.1 ml volume were rubbed into the shaven dorsal skin of mice. The same percutaneous treatment was repeated at the same site after a 2 week interval. The reaction was elicited 28 days after the beginning of the treatment by rubbing 0.25 μg DNCB into the skin. Readings were made 24 hours after administering the eliciting dose by recording the largest and the smallest diameter of the reaction area.

Inverse passive Arthus phenomenon was elicited in *guinea pigs*. Two mg of ovalbumin were injected intrajugularly, then after a 15 minute interval the following substances were injected into four different sites of the shaven dorsal skin: 0.1 ml normal rabbit serum, 70, 130 and 240 μg antibody nitrogen in 0.1 ml volumes. Development of the reaction was recorded after ½, 2 and 4 hours. After 24 hours the animals were sacrificed and the reaction was read on the inner surface of the dorsal skin by considering the size of the area (erythema, oedema, haemorrhagia) and the intensity of haemorrhagia ($+$ = mild; $++$ = medium; $+++$ = severe).

Hypothermia. Mice were cooled as described by GIAJA [4]. Guinea pigs were anaesthesized with Intranarcon (0.08 g/kg intraperitoneally), then placed in plastic sacs containing lumps of ice. Rectal and skin temperatures were taken by the use of a thermistor. As the skin temperature in normothermic as well as in hypothermic animals was lower by 1.5—3°C than the rectal temperature, in the experimental results only the latter is presented.

Results

Preparation and eliciting of control mice for passive cutaneous anaphylaxis were performed in normothermic condition. In the first series of experiments the antibody dose was given to normothermic and the eliciting antigen dose to deeply hypothermic animals. Then, after 20—30 minutes cooling, the animals were brought gently into normothermic condition. As shown in Table I in hypothermia the reaction almost entirely failed to develop. In the second series of experiments (10 animals) antibody and antigen were injected in deep hypothermia. The same effect was observed: cooling prevented the development of the reaction. In the third series of experiments (10 animals) the antibody was injected in hypothermia, then the animals were warmed up and the antigen was given at normal body temperature. In this experiment the skin reaction developed but it was somewhat less marked than in the control animals.

Table I

Passive cutaneous anaphylaxis in normothermic and in hypothermic mice

Designation of animals	Rectal temperature when eliciting the reaction, °C	Degree of reaction	Designation of animals	Rectal temperature when eliciting the reaction, °C	Degree of reaction
1	37.2	+++	11	17.0	—
2	37.0	++	12	17.5	±
3	36.8	++	13	16.5	—
4	37.0	+++	14	17.0	—
5	36.8	++	15	15.5	—
6	37.2	+++	16	16.0	±
7	37.1	+++	17	16.0	—
8	37.0	+++	18	17.0	±
9	37.2	+++	19	16.0	—
10	36.9	++	20	16.5	—

As mice cooled by GIAJA's method reach very rapidly a deep hypothermic state, in the fourth series of experiments (10 mice) both antibody and antigen were given to normothermic animals which were then cooled rapidly. However, under these conditions hypothermia exerted no inhibitory effect on passive cutaneous anaphylaxis.

In hypersensitivity experiments it was examined whether hypothermia influenced the reaction elicited by DNCB. The results are shown in Table II.

Similarly to passive cutaneous anaphylaxis DNCB hypersensitivity was not inhibited by hypothermia if cooling was performed at the time of sensitization (10 animals) or immediately after giving the eliciting dose (10 animals).

Table II

DNCB hypersensitivity reaction in normothermic and in hypothermic mice

Designation of animals	Rectal temperature when eliciting the reaction, °C	Reaction*	Designation of animals	Rectal temperature when eliciting the reaction, °C	Reaction*
			33	19.0	—
21	36.5	10/6	34	18.5	—
22	37.2	15/6	35	20.0	4/3
23	36.8	20/5	36	19.0	—
24	36.5	16/8	37	19.4	—
25	36.5	13/5	38	19.5	—
26	37.1	12/4	39	20.1	—
27	36.4	9/6	40	18.7	—
28	37.0	7/7	41	20.3	5/3
29	37.0	6/2	42	19.2	—
30	36.8	9/6	43	19.0	—
31	20.0	3/3	44	19.0	—
32	19.7	—	45	18.4	—

* Diameter of inflammated area in mm (largest/smallest).

It is known that guinea pigs tolerate cooling less easily than mice and in the former the lowest limit of hypothermia is about 20°C. As shown in Fig. 1 in control normothermic animals 70 μg antibody caused a moderate

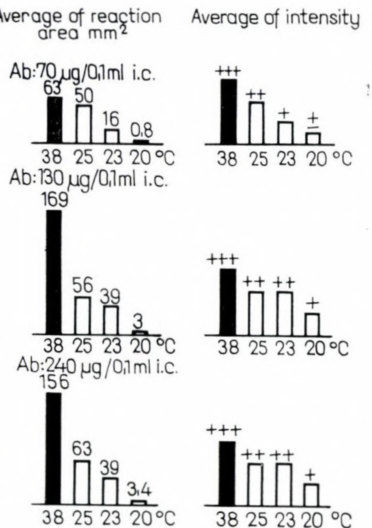

Fig. 1. Inverse passive Arthus reaction in guinea pigs. Figures under the columns indicate body temperature of the animals. Average of data obtained in 10 animals for each experiment. Reaction area in mm²

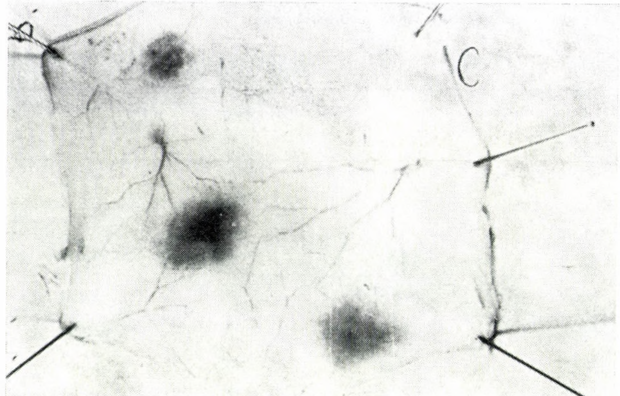

Fig. 2. Inverse passive Arthus reaction in guinea pigs. Rectal temperature when eliciting the reaction, 38.2°C

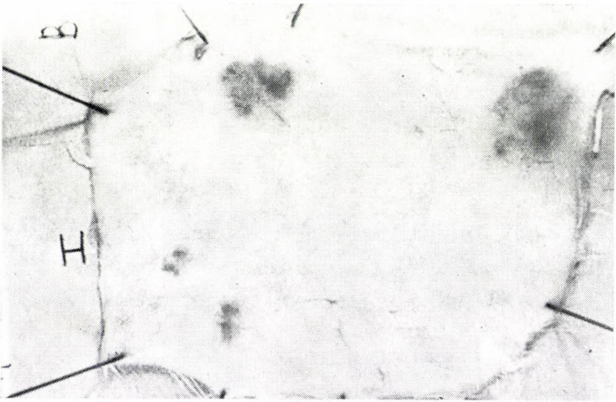

Fig. 3. Inverse passive Arthus reaction in guinea pigs. Rectal temperature when eliciting the reaction, 25.3°C

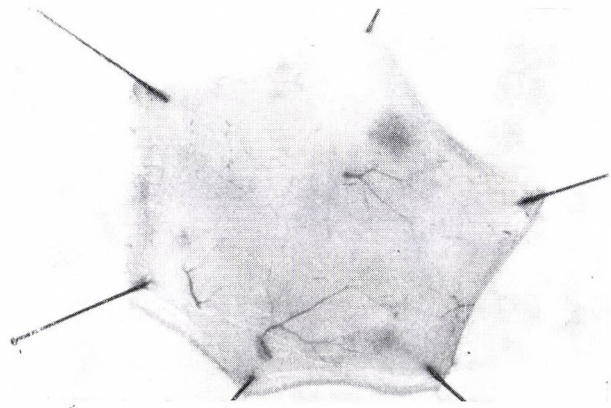

Fig. 4. Inverse passive Arthus reaction in guinea pigs. Rectal temperature when eliciting the reaction, 23.1°C

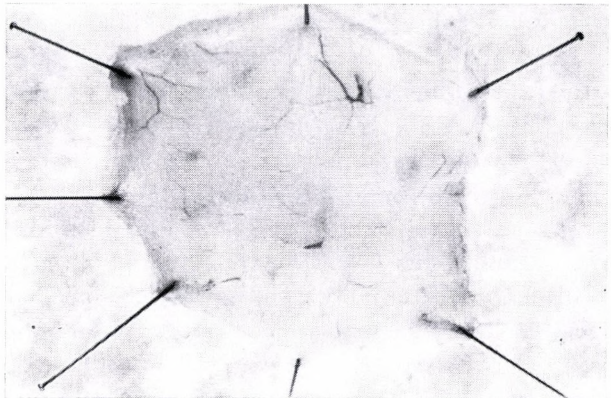

Fig. 5. Inverse passive Arthus reaction in guinea pigs. Rectal temperature when eliciting the reaction, 20.2°C

reaction, but 130 and 240 μg doses gave rise to definite changes. In hypothermic animals the size of the reaction area as well as the intensity of the reaction decreased with the lowering of the body temperature and at 20°C no inverse passive Arthus phenomenon developed (Figs 2—5).

Discussion

Our experimental results indicate that allergic-type skin reactions can be totally or markedly inhibited by cooling the animals. The data show that hypothermia exerts the most definite protective effect if the animals are in hypothermic condition at the time of eliciting the reaction. We described a similar observation in anaphylactic shock [1].

In a previous paper we described that in hyperglycaemia due to alloxan treatment skin reactions develop less readily than in animals with normal blood sugar level [5]. In view of these findings the blood sugar level of hypothermic animals was checked regularly in the present experiments. As the maximum value was 135 mg%, it has been assumed that cooling is never associated with hyperglycaemia high enough to influence the development of skin reactions.

UTCHITEL and KRIMSKI [6] in studying the effect of hypothermia on the Arthus reaction have concluded that corticosteroids released during cooling are responsible for the inhibitory effect. Our experimental findings do not confirm this hypothesis, as in animals cooled in general anaesthesia very slight amounts of corticosteroids are mobilized [7] and cortisone exerts no appreciable effect on passive cutaneous anaphylaxis [8] or on passive Arthus reaction [9].

Hypothermia markedly inhibits the disruption of mast cells [10] and the release of histamine [11]. According to HAYASHI *et al.* [12] histamine plays a role in the first rapid, humoral ("anaphylactic") phase of Arthus phenomenon but it exerts no effect on the reaction itself. ÓVÁRY and BIER [13] have shown that specific antihistamines inhibit the development of passive cutaneous anaphylaxis. Recently LIEBERMAN and ÓVÁRY [14] have shown that histamine plays an important role in passive cutaneous anaphylaxis elicited either by homologous or by heterologous antibodies. In our opinion hypothermia, especially in passive cutaneous anaphylaxis, inhibits the development of the reaction by decreasing the release of histamine.

In our earlier experiments [15] we showed that in hypothermia the degree of antigen-antibody reaction decreases considerably. KING and FRANCIS [16] demonstrated that cooling of organs *in vitro* decreases the degree of tissue antibody fixation and the antigen-antibody reaction takes place under this condition more slowly than at normal temperature. Further experiments are needed to elucidate the alteration of antigen-antibody reaction *in vivo*.

From the experiments of URIUHARA and MOVAT [17] the mechanism of Arthus phenomenon can be summarized as follows. Polymorphonuclear leucocytes accumulating at the site of the reaction phagocytize the antigen-antibody complexes, upon the effect of which the lysosomes are degranulated. For the characteristic changes the protease-type enzymes of lysosomes are responsible. It is still debated whether a similar mechanism is involved in passive cutaneous anaphylaxis [14]. Our results [18] indicated that hypothermia highly inhibited the release of lysosomal enzymes. In subsequent experiments we wish to prove this effect also in allergic-type reactions.

DNCB hypersensitivity is a delayed reaction and we know little of its mechanism. According to WILLOUGHBY *et al.* [19], similarly to tuberculin reaction, the permeability factor released from mononuclear cells plays an important role in DNCB reaction. The effect of this factor is not influenced by histamine or serotonin antagonists. It may be supposed that hypothermia inhibits the release or perhaps the effect of this permeability factor.

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COMPARATIVE STUDIES ON THE TAXONOMIC RELATIONSHIP BETWEEN MYCOBACTERIUM ABSCESSUS AND MYCOBACTERIUM BORSTELENSE

INCIDENCE OF MYCOBACTERIUM ABSCESSUS IN MONKEYS*

By

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Summary. (i) On the basis of biochemical, pathogenic and serological properties *Mycobacterium abscessus* is a well defined species not related antigenically to *Mycobacterium fortuitum* and should not, therefore, be regarded as a subspecies of the latter. *M. abscessus* belongs to the serological group D of mycobacteria.

(ii) *M. abscessus* and *Mycobacterium borstelense* are identical in biochemical properties and antigenic structure but differ in mouse pathogenicity. Therefore they may be classified as two separate species belonging to the same serological group.

(iii) Strains received as *Mycobacterium runyonii* were in all characters identical with *M. abscessus.*

(iv) Strains M4 and M15 isolated from *Macacus rhesus* monkeys were identical with *M. abscessus* in biochemical, pathogenic and serological properties. In the guinea pig strain M15 failed to induce immunity against tuberculosis, but exerted a sensitizing effect against infection by virulent tubercle bacteria.

An increasing number of observations indicate that *Mycobacterium abscessus* may be associated with human infections. MOORE and FRERICHS [11] isolated on repeated occasions a rapidly growing mycobacterium from a patient suffering from arthritis with abscess. They named the new mycobacterial species *M. abscessus.* BOJALIL, CERBÓN and TRUJILLO [3] studied 5 mycobacterial strains isolated from human infections in the United States, Cuba and Mexico and named them *Mycobacterium runyonii.* TSUKAMURA, TSUKAMURA and MIZUNO [15] performed numerical taxonomic analysis of the strains described by BOJALIL *et al.* as *M. runyonii* and of some strains isolated in Japan and classified them as a subspecies of *Mycobacterium fortuitum.* Later TSUKAMURA, MIZUNO and TSUKAMURA [16] revealed that *M. runyonii* was identical with *M. abscessus,* but they still regarded the organism as *M. fortuitum* subs. abscessus. On the basis of biochemical, sensitin and numerical taxonomic studies TAKEYA, NAKAYAMA and NAKAYAMA [14] were of the opinion that *M. runyonii* belonged to a species different from *M. fortuitum.* Their own isolates as well as strains obtained from various sources as *M. fortuitum* showed the properties of *M. runyonii.* MAGNUSSON, ENGBEAK and

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Bentzon [8] examined the specific sensitins of human mycobacterial strains "29" and "66". These cultures were later identified by Magnusson [9] as *M. abscessus*. Stanford and Beck, as mentioned by Runyon [13] and Pattyn [12] isolated rapidly growing strains from skin ulcer and abscess; these strains were identified as *M. abscessus*. Hosty [4] demonstrated the same organism in pulmonary disease. Bönicke [1] described 15 fast-growing mycobacteria isolated from humans as a separate species, *M. borstelense*. As shown by Bönicke and Stottmeier [2] part of these strains produced high amounts of niacin (*M. borstelense* var. niacinogenes). The close relationship between *M. borstelense* and *M. abscessus* was indicated by the biochemical studies of Käppler [5]. Karasseva, Weiszfeiler and Krasznay [7] isolated from *Macacus rhesus* monkeys two fast-growing mycobacterial strains ("4" and "15") not examined so far for relationship to the above-mentioned mycobacterial species.

In the present study the taxonomic relationship between *M. abscessus*, *M. runyonii*, *M. borstelense* and strains M4 and M15 as well as serological connections between these organisms and *M. fortuitum* were examined.

Materials and methods

Strains. M. abscessus ATCC 19977, *M. runyonii* ATCC 14472 (SB 32), *M. runyonii* La 2408, *M. borstelense* SN 281 and 922, *M. borstelense* var. niacinogenes SN 286 abd 923, *M. abscessus* M4 and M15 [6], *M. fortuitum* ATCC 6841.

Properties of the cultures (colony morphology, growth at different temperatures, niacin content, enzyme activity) were examined as described by Meissner [10] and Käppler [5].

Virulence was tested in albino mice by intravenous injection of 1 mg bacteria. Surviving animals were sacrificed 30—60 days after infection and pathological lesions in their organs were examined. Cultures from the organs were made on Löwenstein—Jensen medium.

Antigenic structure was examined by Ouchterlony's gel precipitation method as described by Weiszfeiler *et al.* [18]. Anti-abscessus and anti-fortuitum sera were prepared in rabbits with *M. abscessus* M15 and *M. fortuitum* ATCC strain, respectively.

Immunizing capacity against tuberculosis was examined by injecting subcutaneously 10 guinea pigs with a dose of 1 mg of *M. abscessus* strain M4. One month after immunization the test group and the control group consisting of 10 animals were challenged by injecting 0.0001 mg H37Rv culture subcutaneously.

Three months after infection the animals were sacrificed and specific changes in the organs were expressed by Weiszfeiler's index [17].

Results

All the examined 7 strains grew abundantly on Löwenstein—Jensen medium after 3 days. The colonies were smooth, white or pink in colour. *M. runyonii* SB 32 and *M. borstelense* 286 produced partly R colonies. Biochemical properties of the strains are shown in Table I. *M. abscessus*, *M. runyonii* and *M. borstelense* were identical in biochemical properties. *M. borstelense* strains 286 and 923 (both var. niacinogenes) were niacin positive. *M. fortuitum* differed in amidase spectrum, nitrate reductase, lipase, beta-esterase and

phosphatase reaction from the above strains. On Löwenstein—Jensen medium containing 1000 μg PAS per ml the strains grew well and formed black colonies (blacking phenomenon) in one day (Table I).

Table I

Biochemical reactions

Mycobacterial strains	Niacin	Catalase	Nitrate reductase	Lipase	Arylsulphatase	Phosphatase	Esterase,	Esterase, β	Acetamidase	Urease	Nicotinamidase	Pyrazineamidase	Allantoinase	PAS 1000 μg/ml, blacking
M. abscessus	—	+	—	—	+	—	—	+	—	+	+	+	—	+
M. runyonii SB32	—	+	—	—	+		—	+	—	+	+	+	—	+
M. runyonii 2408	—	+	—	—	+	—	—	+	—	+	+	+	—	—
M. borstelense 281	—	+	—	—	+	—	—	+	—	+	+	+	—	+
M. borstelense 286	+	+	—	—	+	—	—	+	—	+	+	+	—	+
M. borstelense 922	—	+	—	—	+	—	—	+	—	+	+	+	—	+
M. borstelense 923	+	+	—	—	+	—	—	+	—	+	+	+	—	+
Mycobacterium M4	—	+	—	—	+	—	—	+	+	+	+	+	—	+
Mycobacterium M15	—	+	—	—	+	—	—	+	+	+	+	+	—	+
M. fortuitum ATCC 6841	—	+	+	+	+	+	—	—	+	+	+	+	+	—

Pathogenicity of the strains to albino mice and the results of cultural examinations are shown in Table II.

M. abscessus, M. runyonii, strain M4 and strain M15 caused severe alterations in the kidney and hypertrophy of the spleen. While the *M. abscessus* strain gave rise to mild changes, its subculture ("955") isolated from the infected animal was highly pathogenic and caused the death, due to severe alterations in the kidney, of many animals as soon as 2—7 days after injection. From the organs of the animals the bacteria grew in dense culture 60—90 days after infection. None of the 10 mice infected with *M. borstelense* died during the 60 day observation period, their organs were free from pathological changes and cultural examinations were negative.

Gel precipitation with immune serum for strain M15 revealed 6 precipitation lines against antigens prepared from the homologous culture and from strain M4 (Figs 1 and 2). The antigenic identity of strains M15 and *M. runyonii* 14472 was also demonstrated (Fig. 2). Antigens for *M. fortuitum, M. smegmatis, M. phlei*, and *M. tuberculosis* H37Rv showed no precipitation with antiserum M15. A weak line was observed with *M. avium* antigen and

Table II

Pathogenicity tests

Strains	No. of animals	Animals died		Cultural examination		Pathological changes in organs			
		No.	After days	Days after infection	Intensity of growth	Lung	Liver	Spleen	Kidney
M. abscessus 19 977	10	4	28—35	60	++	—	—	±	±
M. borstelense SN 286	5	—	—	60	—	—	—	—	—
M. borstelense SN 281	5	—	—	60	—	—	—	—	—
M. runyonii La 7408	10	—	—	—		—	—	+	+++
M. runyonii 14 472	10	3	23	90		—	—	+	+++
M. abscessus M 4	10	6	67—90	90	+++	—	—	+	++++
M. abscessus M 15	10	6	13—21	51	+++	—	—		++++
Strain 955	10	10	2—7			+++	—	—	—

Table III

Gel precipitation reactions with unabsorbed and absorbed M15 serum

Immune serum	absorbed by	Number of precipitation lines				
		M. abscessus		*M. runyonii*	*M. borstelense*	
		M15	ATCC	ATCC	SN 281	SN 286
M. abscessus M15	—	6	6	6	4	4
M. abscessus M15	*M. abscessus* ATCC	—	—	—	—	—
M. abscessus M15	*M. borstelense*	—	—	—	—	—

Table IV

Immunization tests

Strain used for immunization	Number of guinea pigs	Tuberculosis index
M. abscessus M4	5	17.1
Nil	7	13.7

4—6 lines with *M. abscessus* ATCC strain (Figs 3, 4 and 5). *M. abscessus* antigen failed to react with phlei, smegmatis, H37Rv, avium and kansasii sera but showed precipitation with serum M15. *M. borstelense* strains 281 and 286 were antigenically identical with strains M15 and M4 (Figs 5 and 6).

M. fortuitum antiserum precipitated *M. fortuitum* antigen but not M15 antigen (Fig. 7).

Antiserum M15 was absorbed with *M. abscessus* and *M. borstelense* antigens. As shown in Table III the absorbed serum failed to precipitate not only

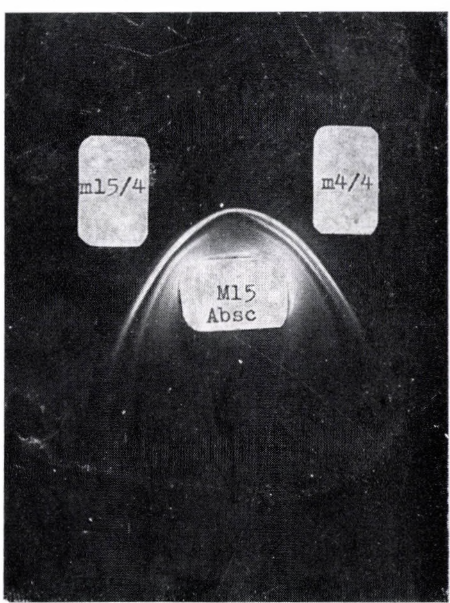

Fig. 1. M. abscessus homologous gel precipitation system. Immune sera are denoted by capital letters: M15 = anti-abscessus M15; F = anti-fortuitum; K = anti-kansasii; P = anti-phlei; S = anti-smegmatis; H_{37} = anti-H37Rv; A = anti-avium ATCC. Antigens are shown by the corresponding small letters: absc = *M. abscessus* ATCC antigen; borst 281 = *M. borstelense* 281 antigen; borst 286 = *M. borstelense* 286 antigen; r = *M. runyonii* ATCC antigen. Denominators indicate the dilution of the sera (1 : 4 or 1 : 32)

the homologous antigen but also *M. abscessus*, *M. runyonii* and *M. borstelense* antigens. These examinations indicated the antigenic identity of strain M15 and the above organisms.

Immunization experiments are summarized in Table IV. The index for tuberculous changes in control animals was 13.7. In mice injected previously with strain M4 and challenged with virulent tubercle bacteria more severe changes expressed by an index of 17.1 were observed.

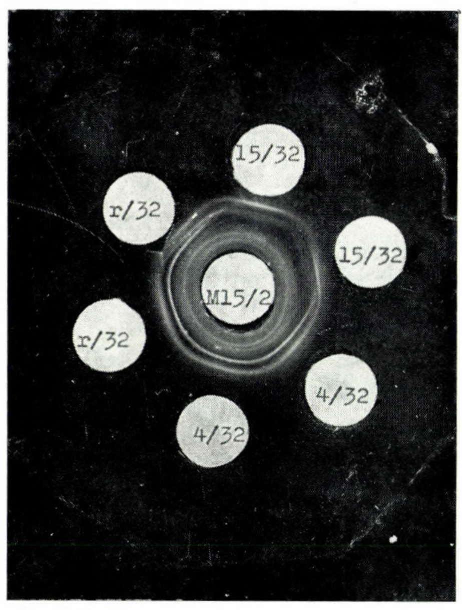

Fig. 2. Central reservoir: *M. abscessus* M15 reference serum diluted 1 : 2. See legend of Fig. 1

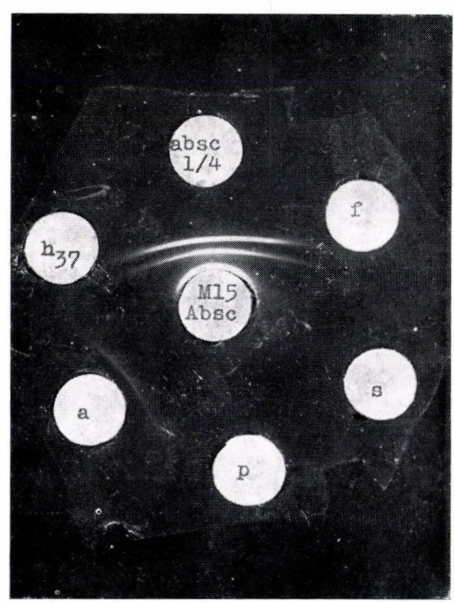

Fig. 3. Central reservoir: *M. abscessus* M15 serum. Peripheral reservoirs: antigens as indicated in Fig. 1

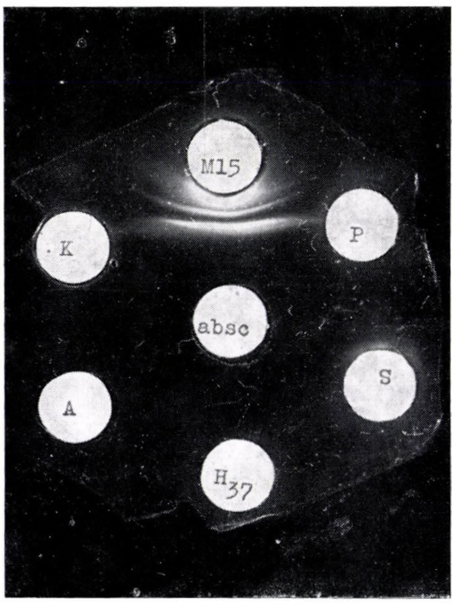

Fig. 4. Central reservoir: *M. abscessus* ATCC antigen. Peripheral reservoirs: reference sera as indicated in Fig. 1

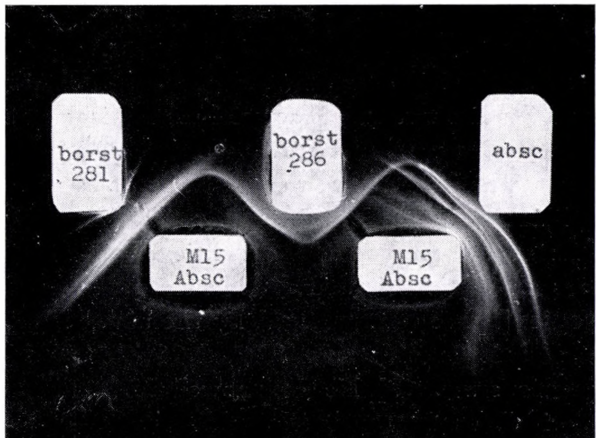

Fig. 5. Lower reservoirs: *M. abscessus* M15 serum. Upper reservoirs: antigens as indicated in Fig. 1

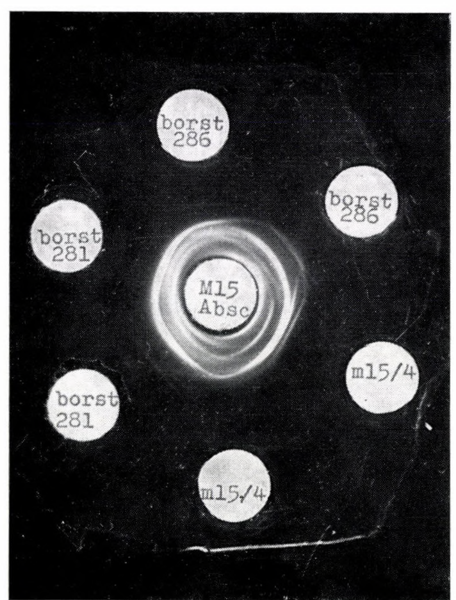

Fig. 6. Central reservoir: *M. abscessus* M15 serum. Peripheral reservoirs: antigens as indicated in Fig. 1

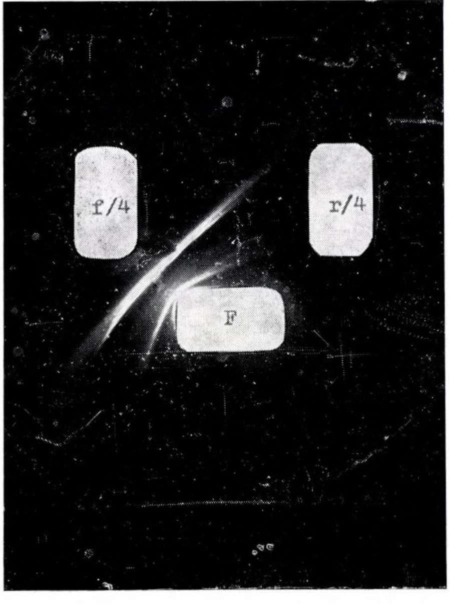

Fig. 7. Lower reservoir: *M. fortuitum* serum. Upper reservoirs: antigens as indicated in Fig. 1

Discussion

In the present studies two mycobacterial strains isolated from monkeys were studied. Both strains were fast-growing organisms, that is saprophytic in cultural character, which, however, were pathogenic to mice. These strains were identified by KÄPPLER [6] as *M. runyonii* described by BOJALIL *et al.* Our studies have confirmed this finding as the identical precipitation line pattern and complete cross absorption showed the identity of *M. abscessus* and M15 antigens. MAGNUSSON [9] has demonstrated by the use of his sensitin method that strain M15 is identical with strains 29 and 66 isolated in Denmark and described in his previous work [8]. He also showed the identity of these strains and cultures described as *M. runyonii.*

Since TSUKAMURA *et al.* [16] and our present studies have shown the identity of *M. abscessus* and *M. runyonii* [15], the latter cannot be regarded as a new species. Strains isolated by STANFORD and BECK [13] in England from subcutaneous abscesses were also identified as *M. abscessus.* HOSTY [4] isolated this organism from pulmonary disease, PATTYN [12] in Belgium and Congo from cutaneous ulcers. Accordingly, *M. abscessus* is a facultatively pathogenic microorganism occurring in Europe, Asia (Japan), America (U.S.A., Mexico) and Africa (Congo). It has been shown not only in man but also in monkeys.

On the basis of numerical taxonomic studies TSUKAMURA *et al.* regarded *M. abscessus* as a subspecies of *M. fortuitum.* This conception should be rejected since in antigenic structure *M. abscessus* differs from other rapidly growing mycobacteria and thus from *M. fortuitum* so much that we have included it in a separate serological group containing only *M. borstelense.* This consideration does not exclude, of course, the common origin of the two organisms. Deep changes as plurifactorial mutations affecting antigenic structure [17] may lead to the formation of new species. *M. fortuitum* and *M. abscessus* are both pathogenic to mice and multiply mainly in the kidney where they give rise to abscesses. MOORE and FRERICHS [11] failed to demonstrate this effect probably because their strain lost its virulence on serial passages. As demonstrated in this study with strain 955, passage in the mouses restores the high virulence of the organism. It is noticeable that strain 955 caused lethal infection in 2—7 days.

M. abscessus is a facultatively pathogenic microorganism. Soil is probably its natural source and it invades man or animals but occasionally, perhaps through lesions of the skin or through inhalation. It is, however, remarkable that *M. abscessus* may spread from artificially infected to healthy mice (WEISZFEILER 1969). It is a debated problem whether infection by atypica mycobacteria induce immunity against tuberculosis. As to *M. abscessus*, our

studies indicate that infection with this organism aggravates the course of a subsequent tuberculosis infection.

M. borstelense described by Bönicke is very similar in properties to *M. abscessus* according to Käppler [5]. Tsukamura *et al.* described this organism as a separate species. Our studies indicated that in antigenic structure it stands very close to *M. borstelense*. Absorption of *M. abscessus* serum with *M. borstelense* removes all precipitating antibodies for the homologous strain, that is, the two organisms are by this method antigenically identical. In pathogenic properties there is a considerable difference: *M. borstelense* is not pathogenic to mice while *M. abscessus* causes fatal infection with severe changes in the kidney. *M. borstelense* cannot be isolated 60 days after injection from mice, that is, it behaves like a saprophyte. In our opinion the two organisms are closely related and thus they belong into one serological group. It seems likely that many *M. abscessus* strains have been incorrectly determined as *M. fortuitum*.

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INTESTINAL MICROFLORA OF THE LARVAE OF ST. MARK'S FLY

IV. STUDIES ON THE INTESTINAL BACTERIAL FLORA OF A LARVA-POPULATION

By

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Summary. In the intestinal tract of the larvae of St. Mark's fly there is a definite selection of bacterial flora ingested with organic remains and soil matter. The absolute predominant member of the bacterial population comprising more than 70% of the total intestinal microflora was *Pseudomonas fluorescens. Enterobacter aerogenes* occurring infrequently in soil and an intermediary *Enterobacteriaceae* biotype were also important constituents. Some representatives of the soil microflora (*Bacillus cereus, Mycobacterium* sp., *Chromobacterium* sp., etc.) survived the intestinal passage and were recovered in more or less reduced numbers. Others, mainly inhabitants of horizons A_H and A_F (*Bacillus subtilis, Bacillus sphaericus, Alcaligenes faecalis, Achromobacter pestifer, Agrobacterium radiobacter, Rhizobium* spp., *Micrococcus cryophilus*, etc.) were absent from the middle and hind intestine and from fresh excrement. Although this type of selection is characteristic, the qualitative and quantitative composition of the predominant organisms is considerably influenced by environmental factors (food and soil), which may alter the survival chance of soil microflora passing through the digestive canal.

Computer analysis of the intestinal actinomycete flora of *Bibio marci* larvae revealed complicated selection processes taking place in the digestive canal [1]. It has been shown that only some species of the actinomycete population in soil are able to multiply in the intestinal tract of the larvae. In view of the fact that actinomycetes play only a secondary role in the intestine of St. Mark's fly, it seemed important to study these selection processes in relation to enteric bacteria. The investigations were performed on individual and collective intestinal samples taken from 220 larvae of population No. 6 described in our previous paper [2].

Materials and methods

1. *Quantitative examination of intestinal bacterial flora* was performed with specimens taken aseptically from the middle and hind intestine and with fresh excrement by use of the following media: (a) N-free Ashby agar; (b) peptone agar; (c) casein-glucose agar; (d) glucose-asparagine agar; (e) cellulose-peptone agar; (f) cellulose ammonium sulphate agar; (g) glycerolarginine agar. Media (e) and (f) were incubated both aerobically and anaerobically.

2. *Isolation of strains* for identification was performed by transferring colonies from the above media to agar slant (see below).

3. *Pure culture* was obtained by streaking from the stock culture or from dilution series made on peptone-glycerol agar onto plates. Reisolation of the strain was checked by streaking

simultaneously on agar plates. During the examination period the purity of the strains was checked by making smears and plate cultures [3].

4. *Maintenance of strains* was made partly by freeze-drying partly by storing at 4—6°C. Strains maintained on slants were subcultured at intervals of 4 days to 2 weeks according to the viability of the cultures. After inoculation the media were incubated at 28°C for 24—28 hours.

5. *Standard inoculum.* A loopful of culture grown on the stock agar medium for 24—48 hours, or 0.05 ml of one loopful of bacteria suspended in 10 ml saline was used.

6. *Stock agar medium.* Most strains grew well on yeast-extract-glucose agar [4]: yeast extract, 5 g; peptone, 5 g; glucose, 10 g; agar, 20 g; distilled water, 1000 ml; pH 7.2.

7. *Incubation*, except for certain special examinations, was performed at 28°C.

8. *Colony morphology.* Peptone-glycerol agar, stock agar medium and sometimes special media were used. The properties were recorded after 2—6 days incubation as follows: (a) colony type (S, SR, R); (b) edge (undulate, rhizoid, dentated, lobated); (c) elevation (flat, papillary, wrinkled, convex); (d) consistency (butyrous, dry-friable, mucoid, viscid); (e) surface (glistening, matt).

9. *Cell morphology.* (a) Arrangement of cells (filament, chain, cluster, packet); (b) shape according to HARRINGTON's classification [5]: long rods, $> 5\ \mu$; medium rods, $3\ \mu - 5\ \mu$; short rods, $< 3\ \mu$; coccus; oval; branching; (c) presence of spores.

10. *Examination of cell morphology* was performed with 24—48 hour cultures grown on stock agar medium under phase-contrast microscope and in Gram-stained smears. Motility was examined in hanging drops prepared with 2 day peptone water cultures. The method of GEMMEL and HODGKISS [6] elaborated for the macroscopic detection of the motility of lactobacilli was also employed.

11. *Flagella staining and electron microscopic studies.* Flagellae were stained by PEPPLER's tannin method. Electron micrographs were prepared in the Tesla 242A apparatus. Agar medium was overlayered with collodion, seeded by spraying the bacterial suspension on the membrane and incubated until microcolonies developed. Then the membrane was mounted and shadowed with palladium at 20° angle.

12. *Spores* were shown by staining with aniline fuchsin then with methylene blue.

13. *Cultural characters.* In comparing the cultures pigment production on peptone-glycerol agar, dissociation on starch agar medium, etc. were considered. For the detection of characteristic pigments of certain organisms special media were employed.

14. *Growth characters* were examined on peptone-glycerol agar slants incubated at 37°C and 45°C for 48 hours and at 5°C for 1 week.

15. *Heat tolerance.* Twenty-four hour cultures prepared in nutrient broth (meat extract, 3.0 g; peptone, 5.0 g; distilled water, 1000 ml) were exposed in water bath to 56°C and 60°C for 10 minutes. The cultures were then rapidly cooled, subcultured on stock agar slants, incubated for 48 hours and compared with the unheated control.

16. *Influence of pH on growth.* Nutrient broth was adjusted to pH 4.0 with HCl and to 8.0 with NaOH. Observation of the inoculated medium lasted for 6 days.

17. *Salt tolerance* was tested in nutrient broth containing 3.5 and 10% NaCl. Observation lasted for 6 days.

18. *Phenol resistance* was tested as described by SNEATH [7]. Aliquots of 1.6 ml nutrient broth culture were incubated for 48 hours at 28°C. Then 0.4 ml 5% aqueous phenol solution was added to each tube. After exposure at 20°C for 7 minutes 0.05 ml samples were diluted with 5 ml broth. Finally one drop of the suspension was transferred to agar slant and incubated for 4 days.

19. *Indole production.* Three to 6 day peptone water (Bacto peptone, 1%; NaCl, 0.5%; pH 7.2) cultures were extracted with xylene and tested with KOVÁCS's reagent.

20. *Ammonia production* was detected in 4 day peptone water cultures with Nessler reagent.

21. *Arginine hydrolysis.* After incubation for 5 days in arginine-glucose medium (arginine, 3 g; glucose, 1 g; K_2HPO_4, 1 g; yeast extract, 1 g; distilled water, 1000 ml; pH 7.0) ammonia was shown with Nessler reagent.

22. *Arginine dihydrolase* was detected as described by RICHARD [8].

23. *Hydrogen sulphide* production from cysteine, sodium thiosulphate and peptone was shown with lead acetate paper strips. Culture media for the detection of H_2S production from cysteine and thiosulphate consisted of: glycerol, 12 ml; asparagine, 1.5 g; K_2HPO_4, 1.5 g; NaCl, 3.0 g; $MgCl_2$, 0.75 g; $CaCO_3$, 0.3 g; distilled water, 1500 ml. To 1.5 litres of basal medium 0.185 g thiosulphate or 0.283 g L-cysteine was added.

24. *Methyl red* (MR) and *Voges—Proskauer* (VP) reactions were tested in fluid glucose-phosphate medium. Acetoin was detected by BARRIT's method.

25. *Nitrate reduction* was examined in ISP nitrate broth (Difco). Nitrite was shown after 4—12 days incubation with Griess—Ilosvay reagent. Reduction beyond nitrite was tested by the zinc dust method.

26. *Starch hydrolysis* was detected with Lugol solution after 5 days incubation. Basal medium: peptone, 5.0 g; meat extract, 5.0 g; NaCl, 5.0 g; agar, 15.0 g; soluble starch, 4.0 g; distilled water, 1000 ml;

27. *Aesculin hydrolysis* was tested by SNEATH's method [7] in peptone-aesculin-iron citrate medium.

28. *Urease activity* was shown on CHRISTENSEN's agar [9] incubated for 2—7 days.

29. *Lecithinase activity* was tested on egg-yolk agar.

30. *Cellulase activity* was detected by microscopic examination of filter paper strips incubated for 7—14 days in peptone water or ammonium sulphate liquid medium.

31. *Methylene blue reduction.* Basal medium: meat extract, 3 g; tryptone, 5 g; distilled water, 1000 ml. After 24 hours incubation 1 drop of 1% methylene blue solution was added and the medium was incubated further for 1—24 hours.

32. *Proteolytic activity.* Nutrient gelatin stabs and LIESKE's egg-white agar plates [11] were incubated for 17 days. Activity on egg-white agar was expressed as the radius of clear zones.

33. *Haemolytic activity.* Basal medium: meat extract, 5.0 g; peptone, 10.0 g; NaCl, 3.0 g; K_2HPO_4 2.0 g; agar, 17.0 g; distilled water, 1000 ml. To each plate 1 ml defibrinated sheep blood was added. Incubation lasted for 2—4 days.

34. *Antibiotic sensitivity.* Peptone-meat extract agar plates were seeded with 24—48 hour cultures, then the antibiotic discs were placed on the plates and after 24 hours incubation the radius of inhibition zones was measured. The following discs manufactured by the Institute for Serobacteriological Production and Research "Human", Budapest, were used: penicillin (3 IU), oxacillin (10 μg), methicillin (20 μg), chloramphenicol (30 μg), streptomycin (30 μg), oleandomycin (30 μg), tetracycline (30 μg), neomycin (100 μg), polymyxin-B (15 μg), erythromycin (10 μg), superseptyl (sulphonamide derivative, 400 μg), nitrofurantoin (300 μg), chlortetracycline (30 μg), oxytetracycline (30 μg), vancomycin (50 μg), kanamycin (30 μg), spiramycin (30 μg), novobiocin (30 μg).

35. *Oxidative and fermentative breakdown* of glucose and lactose was tested by the method of HUGH and LEIFSON [12].

36. *Oxidase test.* The method of KOVÁCS [13] was used.

37. *Catalase production.* Ten per cent hydrogen peroxide was added to 24—48 hour agar slants and gas production was recorded. An uninoculated slant served as control.

38. *Production of growth inhibitory substances.* The test organisms were seeded on peptone-meat extract agar (peptone, 5.0 g; meat extract, 5.0 g; NaCl, 5.0 g; distilled water, 1000 ml; pH 7.0) and the antagonist strain was either spotted or transferred as agar block culture onto the plate. Test organisms: *E. coli, B. subtilis, Saccharomyces carlsbergensis.*

39. *Tyrosinase activity.* Basal medium: L-tyrosine, 1.0 g; yeast extract (Difco), 1.0 g; NaCl, 8.5 g; agar, 16.0 g; tap water, 1000 ml. Incubation lasted for 24—48 hours.

40. *Casein hydrolysis.* Basal medium: meat extract, 3.0 g; tryptone, 5.0 g; glucose, 10.0 g; agar, 15.0 g; distilled water, 1000 ml; pH 7.0; skim milk, 0.5 ml/Petri dish. Incubation lasted for 2—3 days.

41. *Citrate utilization* was tested on SIMMONS' solid medium.

42. *Lipase activity.* SIERRA's method [14] with Tween-80 substrate was used.

43. *Ornithine decarboxylase* was tested according to the method of RICHARD [8]. Lysine decarboxylase activity was demonstrated as described by MØLLER [15].

44. *Phenylalanine deaminase.* The method of COLLINS [16] was used.

45. *Acid production* from D-galactose, maltose, sucrose, L-arabinose, D-fructose, D-mannitol and glycerol was tested in the medium of HUGH and LEIFSON [12].

46. *Carbohydrate utilization as sole source of carbon.* Basal medium [17]: $(NH_4)_2SO_4$, 2.64 g; KH_2PO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; Difco agar, 20 g; distilled water, 980 ml; pH 7.0; the medium was sterilized by autoclaving at 115°C for 30 minutes. The carbon sources (glucose, fructose, arabinose, rhamnose, xylose, galactose, maltose, mannitol, inositol, glycerol) were Seitz filtered and added at 0.5% final concentrations. The basal medium was used as control. Incubation lasted for 16 days.

47. *Utilization of nitrogen sources.* Basal medium [17]: KH_2PO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; glucose, 3.0 g; glycerol, 2.0 g; Difco agar 20 g; distilled water, 1000 ml; pH 7.0. Nitrogen sources ($NaNO_3$, glycine, L-cystine, L-tryptophan, L-arginine and L-asparagine) were added so that the final nitrogen concentration corresponded to 280 mg per litre. The cultures were inoculated also on nitrogen-free Ashby agar.

Results

From intestinal and excrement specimens seeded on Ashby agar no anaerobic or aerobic nitrogen fixing bacteria were isolated. The results were also negative for anaerobic cellulose-decomposing bacteria. On cellulose agar incubated aerobically only some fungi were occasionally observed. These findings confirmed our previous results [18] that in the intestine of larvae there is neither increase in absolute nitrogen content of the organic material ingested nor a significant decomposition of cellulose. The predominating intestinal organisms require less complex nutrient substances, most of them grow well even on inorganic nitrogen sources, or belong to lactose-splitting *Enterobacteriaceae.* Comparison of colonies on primary plates and properties of the isolates indicated that about 2/3 of the bacterial flora belonged to one single species. Seventy out of 100 strains isolated from the intestinal milieu represented this species. Its importance in respect to the ecology of *Bibio* larvae is clearly understood if it is considered that in mixed gut-content specimens from larval community No. 6 the incidence of the bacteria was higher than 70% of the total intestinal flora. These organisms were Gram negative non-acid-fast and non-sporulating rods producing sometimes filaments, were briskly motile by two or more polar flagella, produced green fluorescent pigment and failed to attack lactose. In Table I three representatives of pseudomonads isolated from the intestine of the larvae (strains B-044, B-067 and B-068) are compared to authentic strains. Strains B-067 and B-068 represented predominant pseudomonads of the digestive canal; B-044 was a representative of a frequent variant. These bacteria corresponded to organisms described a "*Ps. fluorescens* species group" [19] or as "*Ps. fluorescens* species" [20]. Strains *Ps. fluorescens* CCM 1969 (NCIB 3756) was very similar in characters to strains B-067 and B-068 (Table I).

Reactions and intensity of growth were denoted in Table I in two different manners, namely on the one hand as + = positive, (+) = doubtful or weakly positive, — = negative reaction or no growth, on the other as — = negative, 1 = weak, 2 = medium, 3 = strong reaction or abundant growth. The degree of antibiotic sensitivity was recorded similarly. Abbreviations: S = smooth, R = rough, Lo = lobular, F = flat, Wr = wrinkled, Bu = butyrous, Ge = glistening, M = matt, r = limit of opaque zone is not sharp, Al = alkaline reaction. The incidence of pseudomonads in the intestine of lower animals is known from the literature [1]. The high incidence in the intestinal tract of *Bibio* larvae reflects the uniformity of the bacterial flora as it has been observed for actinomycetes. *Ps. aeruginosa* strain CCM 1959 used for comparison was able to grow even at 45°C and showed a strict physiological relationship with LÁNYI's general description of this species [21] based on a large number of isolates. The systematic position and individual species rank [20] of *Ps. ovalis* have not yet been elucidated.

Table I

Comparison of Pseudomonas cultures isolated from the digestive canal of the larvae of St. Mark's fly with authentic strains

	Ps. fluorescens CCM 1969 (NCIB 3756)	*Ps. fluorescens* B-044	*Ps. fluorescens* B-067	*Ps. fluorescens* B-068	*Ps. ovalis* CCM 1977 (ATCC 8209)	*Ps. aeruginosa* CCM 1959 (NCIB 6750)
Peptone-glycerol agar						
Colony type	S	S	S	S	R	R
Edge of colony	Lo	Lo	Lo	Lo	(Lo)	Lo
Elevation of colony	F	F	F	F	Wr	F
Consistency	Bu	Bu	Bu	Bu	Bu	Bu
Surface	Ge	Ge	Ge	Ge	M	M
Soluble pigment	—	—	—	—	—	—
Colour of colony	—	—	—	—	—	—
Green fluorescent pigment	+	+	+	+	—	+
Oxidase (Kovács)	3	3	2	2	3	3
Lecithinase	3r	1	3r	3r	1	3
Lipase (Tween-80)	—	—	3	3	—	3
Aesculin hydrolysis	—	—	—	—	—	—
Starch hydrolysis	—	—	—	—	—	—
Casein hydrolysis	2	1	3	3	2	2
Arginine hydrolysis	3	±	3	3	3	3
Ammonia from peptone	3	1	3	3	3	1
Citrate (Simmons)	1, Al	1, Al	1, Al	1, Al	1, Al	1, Al
Haemolysis	1	1	2α	—	1	1
Litmus reduction	+	—	+	+	—	+
Phenylalanine deaminase	—	—	—	—	—	—
Taurocholate	3	3	3	3	3	3
Acid production						
D-Galactose	+	+	+	+	+	+
Maltose	—	—	—	—	—	—
Sucrose	—	—	—	—	—	—
L-Arabinose	+	+	+	+	+	+
D-Fructose	(+)	—	—	—	—	—
D-Mannitol	—	+	—	—	—	(+)
Glycerol	(+)	—	—	—	—	—
Resistance to phenol	—	—	—	—	—	—
Heat tolerance, 56—60°C	—	—	—	—	—	—
pH tolerance, 4.0	—	—	—	—	—	—
8.0	3	3	3	3	3	3

Table I continued

	Ps. fluorescens CCM 1969 (NCIB 3756)	*Ps. fluorescens* B-044	*Ps. fluorescens* B-067	*Ps. fluorescens* B-068	*Ps. ovalis* CCM 1977 (ATCC 8209)	*Ps. aeruginosa* CCM 1959 (NCIB 6750)
NaCl tolerance 3.5%	3	1	3	3	2	1
10.0%	—	—	—	—	—	—
H_2S production						
Cysteine	1	3	—	—	—	—
$Na_2S_2O_3$	—	—	—	—	—	—
Peptone	—	—	—	—	—	—
Tresner—Danga reaction	—	—	—	—	—	—
Gram staining	—	—	—	—	—	—
Ziehl—Neelsen staining	—	—	—	—	—	—
Spores	—	—	—	—	—	—
Motility	+	+	+	+	+	+
MR	—	—	—	—	—	—
VP	—	—	—	—	—	—
Indole	—	—	—	—	—	—
Proteolytic activity						
Gelatin	—	—	—	1	—	2
Egg-white agar	—	—	1	1	—	1
Cellulase	—	—	—	—	—	—
Nitrate reduction						
to nitrite	+	—	+	+	—	+
beyond nitrite	+	—	+	+	—	+
Glucose breakdown						
oxidative	+	+	+	+	+	+
fermentative	—	—	—	—	—	—
Lactose breakdown						
oxidative	—	—	—	—	—	—
fermentative	—	—	—	—	—	—
Methylene blue reduction	3	2	3	3	3	3
Urease	3	±	3	3	2	3
Growth 5°C	3	2	3	3	2	—
37°C	±	±	3	3	±—1	3
45°C	—	—	—	—	—	2
Inhibition of the growth of						
E. coli	—	—	—	—	—	—
B. subtilis	—	1	1	1	1	2
Sacch. carlsbergensis	1	2	2	2	1	3

Table I continued

	Ps. fluorescens CCM 1969 (NCIB 3756)	*Ps. fluorescens* B-044	*Ps. fluorescens* B-067	*Ps. fluorescens* B-068	*Ps. ovalis* CCM 1977 (ATCC 8209)	*Ps. aeruginosa* CCM 1959 (NCIB 6750)
Catalase	3	3	3	3	3	3
Tyrosinase	—	—	—	—	—	—
Antibiotic sensitivity						
Penicillin	—	—	—	—	—	—
Oxacillin	—	—	—	—	—	—
Methicillin	—	—	—	—	—	—
Chloramphenicol	±	±	—	—	—	—
Streptomycin	1	1	1	1	1	1
Oleandomycin	—	—	—	—	—	—
Tetracycline	1	1	1	1	1	1
Neomycin	1	1	1	1	1	1
Polymyxin-B	1	1	1	1	1	1
Erythromycin	—	—	—	—	—	—
Superseptyl	1	1	1	1	±	—
Nitrofurantoin	—	—	—	—	—	—
Chlortetracycline	1	1	1	1	1	1
Oxytetracycline	1	1	1	1	1	1
Vancomycin	—	—	—	—	—	—
Kanamycin	1	1	1	1	1	1
Spiramycin	—	—	—	—	—	—
Novobiocin	—	—	—	—	—	—
Carbon source utilization						
Glucose	1	1	1	1	1	1
Fructose	1	1	1	1	1	1
Arabinose	—	1	—	—	—	—
Rhamnose	—	—	—	—	—	—
Xylose	—	—	—	—	—	—
Galactose	1	1	1	1	1	1
Maltose	—	—	—	—	—	—
Mannitol	1	2	—	—	—	1
Inositol	±, 1	—	—	—	—	—
Glycerol	1	2	1	1	1	1
Negative control (without C)	—	—	—	—	—	—
Positive control (peptone)	3	3	3	3	3	3
Nitrogen source utilization						
$NaNO_3$	2	—	2	2	—	1

Table I continued

	Ps. fluorescens CCM 1969 (NCIB 3756)	*Ps. fluorescens* B-044	*Ps. fluorescens* B-067	*Ps. fluorescens* B-068	*Ps. ovalis* CCM 1977 (ATCC 8209)	*Ps. aeruginosa* CCM 1959 (NCIB 6750)
Glycine	2	3	3	3	2	2
L-Cystine	—	2	—	—	—	—
L-Tryptophan	1	—	—	—	—	—
L-Asparagine	2	3	2	2	2	2
L-Arginine	2	3	3	3	2	2
Negative control (without N)	—	—	—	—	—	—

In lack of suitable methods the percentage incidence of other species of the intestinal flora was not determined. However, we give an account of the approximate frequency of these organisms. ***Enterobacter*** (Aerobacter) ***aerogenes*** occurred much less frequently than *Ps. fluorescens*. The properties of *E. aerogenes* strain B-014 chosen as a representative of the isolates were as follows: $0.3-0.6\times0.5-1.4\,\mu$ motile rods arranged singly or in pairs, sometimes in short filaments and chains. Electron micrography indicated the presence of peritrichate flagellae. IMViC reaction $--++$ (characteristic of the species); oxidase, lecithinase, lipase, phenylalanine deaminase, tyrosinase, cellulase, proteolytic activity and starch hydrolysis negative; casein hydrolysed weakly; nitrates reduced to nitrite; methylene blue reductase, catalase and urease positive; oxidative and fermentative breakdown of glucose and lactose; hydrogen sulphide produced from cysteine, peptone and sodium thiosulphate [1]; Tresner—Danga negative; intense ammonia production from peptone and arginine; weak haemolysis; aesculin rapidly hydrolysed; litmus not reduced; milk strongly acidified; abundant growth and acid production on MacConkey agar; acid production from D-galactose, maltose, L-arabinose, D-fructose, D-mannitol, glycerol, but not from sucrose. Killed at 56°C, not resistant to phenol; growth at pH 8.0 but not at 4.0; 3.5% NaCl well tolerated but no growth at 10% NaCl; growth at 5°C and 37°C but not at 45°C; arginine dihydrolase positive, no dehydrogenation, nucleus or chain breakdown of steroids; growth of *B. subtilis* and *Sacch. carlsbergensis* inhibited but no effect on *E. coli*. Resistant to penicillin, oxacillin, methicillin, oleandomycin, erythromycin, superseptyl, vancomycin, spiramycin and novobiocin, moderately sensitive to chloramphenicol, streptomycin, tetracycline, neomycin, polymyxin-B, nitrofurantoin, chlortetracycline, oxytetracycline, and kanamycin. Utilizes glucose, fructose, arabinose, rhamnose, xylose, galactose, maltose, mannitol

but not inositol as sole source of carbon. Utilizes well glycine, L-asparagine and L-arginine, weakly L-cystine but not L-tryptophan and $NaNO_3$ as nitrogen source. Produces on peptone-glycerol agar S-type, mucoid, colourless colonies with undulate edge and glistening surface; produces no soluble pigment; Gram negative, non-sporulating, non-acid-fast rods.

In addition to *E. aerogenes* an as yet unidentified organism comprised a characteristic although less frequently occurring member of the intestinal flora. The representative strain, B-061, showed the following properties: glucose rapidly fermented; lactose not attacked aerobically or anaerobically; IMViC test — (+) — —; oxidase, lecithinase, lipase, phenylalanine deaminase, tyrosinase, cellulase, proteolytic activity negative; casein and starch hydrolysed; weak catalase activity; nitrate reduced to nitrite; urease negative; methylene blue weakly reduced; hydrogen sulphide produced from cysteine but not from sodium thiosulphate and peptone; Tresner—Danga negative; ammonia not produced from peptone or arginine; haemolysis nil; aesculin rapidly hydrolysed; litmus milk unchanged; good growth but no acid production on MacConkey agar; acid produced from D-galactose, maltose, sucrose, and glycerol but not from L-arabinose, D-fructose and D-mannitol. Resistant to phenol and tolerates 56°C but not 60°C, grows at pH 8.0 but not at 4.0; tolerates 3.5% but not 10% NaCl; grows well at 5°C but not at 37°C; no dehydrogenation, nucleus or chain breakdown of steroids; exerts no definite antibiotic effect; resistant to nitrofurantoin, weakly sensitive to penicillin, oxacillin, methicillin, oleandomycin, polymyxin-B, erythromycin, kanamycin and spiramycin; moderately sensitive to chloramphenicol, streptomycin, tetracycline, neomycin, superseptyl, chlortetracycline, oxytetracycline, vancomycin and novobiocin; grows only on complex media and fails to utilize glucose, fructose, arabinose, rhamnose, xylose, galactose, maltose, mannitol, inositol and glycerol as carbon source; fails to grow at the expense of inorganic nitrogen and to utilize amino acids as nitrogen sources; on peptone-glycerol agar produces easily emulsifiable S-type colonies light yellow in colour with undulate edge and flat glistening surface; forms no soluble pigment, produces no spores, Gram negative and non-acid-fast. This organism belonged neither to *Escherichia* nor to *Enterobacter*. We regard it as an intermediary organism of an as yet undetermined taxonomic position. In Fig. 1 it is designated as "Paracolobactrum".

In addition to organisms discussed above, 4 different species of bacteria were encountered. From their constant presence in low numbers it has been concluded that — being common inhabitants of soil — they are relatively resistant to the selective effect of intestinal passage and their populations pass through the intestine more or less unchanged and without playing any important role. Isolates of this group of organisms belonged to *Bacillus cereus* [22]. The characters of the culture representing these isolates (strain B-062) and of authentic *B. cereus* cultures are shown in Table II.

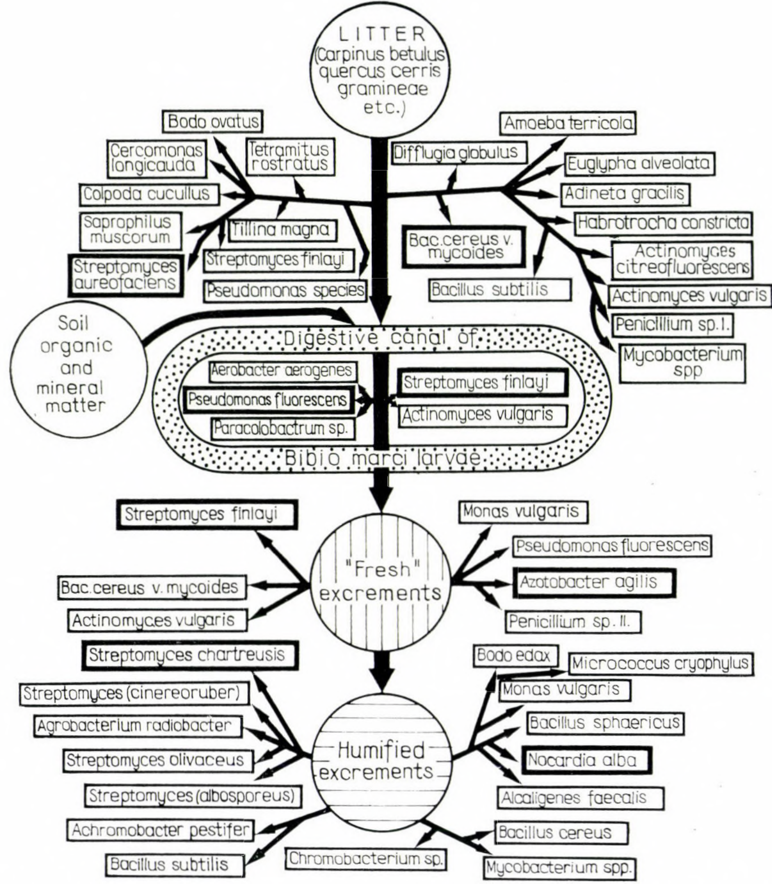

Fig. 1. Succession of microbial communities as an effect of a *Bibio marci* larva-population in the Förna of a mull like forest rendzina

It is evident that strain B-062 belongs to the species *B. cereus*. It is interesting that *B. cereus* var. mycoides occurred frequently in the soil but was absent from intestinal and excrement specimens.

Strains belonging to one *Mycobacterium*, one *Chromobacterium* and one *Brevibacterium* species were isolated occasionally. The *Chromobacterium* strains were not identical with *C. violaceum* and *C. lividum* described by Sneath [7] and Moffet and Colwell [23]. As these organisms occur in horizon A_H and even in the rhizoplane of certain plants, they will be discussed in the general description of the bacterial flora of rendzina soil [24].

As the identification of many soil mycobacteria presents extreme difficulties, only the characters of strain B-047, representative of the *Mycobacterium* species isolated from the intestinal specimens are given: glucose and lactose are attacked neither oxidatively nor fermentatively; oxidase, lecithinase, phenylalanine deaminase, tyrosinase, cellulase negative; lipase positive; no

Table II

Comparison of Bacillus cereus strains

	B. cereus B-062	*B. cereus* OKI 100 002 (IP 5257)	*B. cereus* RIPP, A-115	*B. cereus* var. mycoides RIPP, A-111
Peptone-glycerol agar				
Colony type	R	R	R	R
Edge of colony	(Un)	(Un)	(Un)	Rh
Elevation of colony	F	F	F	F
Consistency	Dr. sm.	Dr. sm.	Dr. sm.	Dr. sm.
Surface	M	M	M	M
Soluble pigment	– (yellow)	– (yellow)	– (yellow)	– (yellow)
Colour of colony	–	–	–	–
Oxidase (Kovács)	–	–	–	–
Lecithinase	1	1	–	?
Lipase (Tween-80)	2	2	1	2
Aesculin hydrolysis	3	2	3	2
Starch hydrolysis	3	3	3	3
Casein hydrolysis	2	2	2	2
Arginine hydrolysis	–	–	–	3
Ammonia from peptone	±	1	±	3
Citrate (Simmons)	–	± –1, Al	3, Al	–
Haemolysis	3β	2β	3β	1
Litmus reduction	+	+	+	+
Phenylalanine deaminase	–	–	–	–
Taurocholate	–	–	–	–
Acid production				
D-Galactose	–	(+)	–	–
Maltose	+	(+)	+	+
Sucrose	(+)	–	–	–
L-Arabinose	–	–	–	–
D-Fructose	(+)	+	+	+
D-Mannitol	–	–	–	–
Glycerol	–	–	–	(+)
Resistance to phenol	3	3	3	3
Heat tolerance, 56°C	3	3	3	3
60°C	3	3	3	3
pH tolerance, 4.0	–	–	–	–
8.0	3	2	2	3

Table II continued

	B. cereus B-062	*B. cereus* OKI 100 002 (IP 5257)	*B. cereus* RIPP, A-115	*B. cereus* var. mycoides RIPP, A-111
NaCl tolerance, 3.5%	2	2	2	3
10%	—	—	—	—
H_2S production				
Cysteine	3	3	3	—
$Na_2S_2O_3$	—	—	—	—
Peptone	2	—	—	1
Tresner — Danga reaction	—	—	—	—
Gram staining	+	+	+	+
Spores	+	+	+	+
Motility	+	—	+	—
MR	(+)	—	—	—
VP	(+)	+	(+)	—
Indole	—	—	—	—
Proteolytic activity				
Gelatin	3	3	2	2
Egg-white agar	1	2	1	3
Peptonization of milk	+	+	+	+
Cellulase	—	—	—	—
Nitrate reduction				
to nitrite	+	+	+	+
beyond nitrite	—	—	—	—
Glucose breakdown				
oxidative	+	—	+	?
fermentative	+	+	+	?
Lactose breakdown				
oxidative	—	—	—	—
fermentative	(+)	(+)	(+)	—
Methylene blue reduction	3	3	—	2
Urease	—	—	—	—
Growth 5°C	2	—	—	3
37°C	1	3	2	3
45°C	—	1	±	—
Inhibition of the growth of				
E. coli	—	—	—	—
B. subtilis	1	—	1	—
Sacch. carlsbergensis	±	1	±	±

Table II continued

	B. cereus B-062	*B. cereus* OKI 100 002 (IP 5257)	*B. cereus* RIPP, A-115	*B. cereus* var. mycoides RIPP, A-111
Catalase	3	3	3	3
Tyrosinase	—	—	—	—
Antibiotic sensitivity				
Penicillin	—	—	—	1
Oxacillin	—	—	—	1
Methicillin	2	2	2	1
Chloramphenicol	1	1	1	1
Streptomycin	1	1	1	1
Oleandomycin	1	1	1	1
Tetracycline	2	2	2	2
Neomycin	1	1	1	1
Polymyxin-B	1	1	1	1
Erythromycin	1	1	1	1
Superseptyl	1	2	1	1
Nitrofurantoin	1	1	1	1
Chlortetracycline	2	2	2	2
Oxytetracycline	2	2	2	2
Vancomycin	1	1	1	1
Kanamycin	1	1	1	1
Spiramycin	1	1	1	1
Novobiocin	2	2	2	2
Carbon source utilization				
Glucose	±, 1	±, 1	±	±
Fructose	—	±, 1	—	—
Arabinose	—	—	—	—
Rhamnose	—	—	—	—
Xylose	—	—	—	—
Galactose	—	—	—	—
Maltose	±, 1	±, 1	±	±
Mannitol	—	—	—	—
Inositol	—	—	—	—
Glycerol	—	—	—	—
Negative control (without C)	—	—	—	—
Positive control (peptone)	3	3	3	3
Nitrogen source utilization				
$NaNO_3$	—	—	—	—

Table II continued

	B. cereus B-062	*B. cereus* OKI 100 002 (IP 5257)	*B. cereus* RIPP, A-115	*B. cereus* var. mycoides RIPP, A-111
Glycine	±	2	1	2
L-Cystine	—	—	—	—
L-Tryptophan	—	—	—	—
L-Asparagine	1	2	1	1
L-Arginine	1	2	1	±
Negative control (without N)	—	—	—	—

proteolytic activity; casein and starch not hydrolysed; catalase positive; nitrate reduced to nitrite; urease positive; methylene blue reduction weak; hydrogen sulphide produced from peptone and cysteine but not from sodium thiosulphate; Tresner—Danga negative; ammonia production from peptone weak, from arginine nil; weak haemolysis; traces of aesculin hydrolysis; litmus milk unchanged; abundant growth and acid production on MacConkey agar; acid production from D-fructose, weak acid from D-mannitol; D-galactose, maltose, sucrose, L-arabinose and glycerol not attacked; resistant to phenol; heating at 60°C tolerated; rapid growth at pH 8.0, no multiplication at pH 4.0; 3.5% NaCl tolerated, no growth at 10% NaCl; steroids dehydrogenated, nucleus and chains attacked; antibiotic activity exerted only against yeasts; resistant to oxacillin and methicillin, moderately sensitive to oleandomycin, polymyxin-B, nitrofurantoin, spiramycin, novobiocin, sensitive to penicillin, chloramphenicol, streptomycin, tetracycline, neomycin, erythromycin, superseptyl, chlortetracycline, oxytetracycline, vancomycin and kanamycin; among carbon sources glucose, fructose, mannitol and glycerol utilized well, galactose weakly and rhamnose, arabinose, xylose, maltose and inositol not at all; good growth in defined medium with $NaNO_3$ and different amino acids (glycine L-cysteine, L-tryptophan, L-arginine, asparagine); no growth on Ashby agar; on peptone glycerol agar R-type, at first emulsifiable then dry pink colonies with undulate edge; no soluble pigment; non-sporulating Gram positive, moderately acid-fast rods 0.5—0.7 × 0.5—8.0 μ in size arranged singly, in pairs or in short chains. Strain B-047 belongs to TSUKAMURA's group B of mycobacteria [17]. According to the scheme of CERBON and BOJALIL [25] it falls into "Branch 1" of rapidly growing orange pigment-producing mycobacteria. Of *M. smegmatis*, *M. phlei* and "irregular branch" comprising the above unit, strain B-047 belongs to the latter. It is interesting that in comparing *M. phlei*, *M. smegmatis*, *M. fortuitum* and *M. rhodochrous*, GORDON and MIHM [26] found that only *M. smegmatis* and *M. fortuitum* produced

changes in the colour of MacConkey agar (in 43 and in 96%, respectively). Strain B-047 grew well on MacConkey agar and produced acid.

Finally the description of strain B-057 contributes to data for *Brevibacterium*. The culture, not belonging to any known species of the genus, was characterized as follows; attacks glucose and lactose neither fermentatively nor oxidatively; oxidase, phenylalanine deaminase, tyrosinase, cellulase and lipase negative; lecithinase weakly positive; proteolytic activity and casein hydrolysis negative; hydrolyses starch; catalase positive; nitrate reduction negative; urease negative; reduces methylene blue rapidly; produces hydrogen sulphide from cysteine and peptone but not from sodium thiosulphate; Tresner—Danga negative; produces ammonia from peptone in slight amounts but not from arginine; weak haemolysis; rapid aesculin hydrolysis; reduces litmus; grows on MacConkey agar but fails to produce acid; produces acid from D-galactose, maltose, L-arabinose, D-fructose and glycerol but not from sucrose and D-mannitol; moderately resistant to phenol and tolerates 56°C; grows at pH 8.0 but not at pH 4.0; highest level of NaCl tolerance 3.5%; fails to dehydrogenate steroids and to attack the nucleus and chains; exerts no antibiotic effect; resistant to penicillin, oxacillin, superseptyl, nitrofurantoin, spiramycin; moderately sensitive to methicillin, streptomycin, oleandomycin, polymyxin-B, erythromycin and kanamycin; sensitive to chloramphenicol, tetracycline, neomycin, chlortetracycline, vancomycin and novobiocin; grows only on complex media; fails to utilize inorganic nitrogen sources and amino acids; does not grow in the presence of glucose, fructose, arabinose, rhamnose, xylose, galactose, maltose, mannitol, inositol and glycerol as sole sources of carbon; no growth on N-free Ashby agar; on peptone-glycerol agar produces easily emulsifiable, colourless colonies with undulate edge and glistening surface; produces no soluble pigment; Gram positive, non-acid-fast, nonsporulating rods 0.3—0.5 $\times$ 0.8—4 μ in size arranged singly or in pairs.

The examination of intestinal and excrement specimens confirmed that bacterial species predominant in, and characteristic of, horizons A_H, A_F and A_{00} [24] occur in small numbers or not at all in the intestinal tract of *Bibio* larvae. Thus *B. subtilis*, *B. sphaericus*, *Alcaligenes faecalis*, *Achromobacter pestifer*, *Agrobacterium radiobacter*, *Rhizobium* spp., *Micrococcus cryophilus*, etc., the common inhabitants of rendzina milieu were entirely absent from the fresh excrement of the animals.

The present results, as mentioned in the introduction, reflect the examination of intestinal and excrement specimens taken from larva-population No. 6 [2]. It seemed desirable to perform an orientation analysis in other larva-populations of the rendzina milieu. These examinations will be described in a subsequent paper. However, it is necessary to point out here that the selection of microflora taking place in the intestine can generally be demonstrated, but as to systematic position and quantitative relationship the bacterial species

gaining predominance in the intestine may vary considerably. For the difference many factors as quality of food, physiological state of the animal, composition of soil microflora, etc., may be responsible.

Discussion

The present studies on the intestinal bacterial microflora of *Bibio* larvae indicate a selection similar in mechanism to the selection of intestinal actinomycetes [1, 27]. In course of the intestinal passage of litter and soil matter certain microorganisms, though they may be ingested in very small numbers, become predominant members of the animals' flora. These bacteria are able to multiply rapidly in the digestive canal and play an important role in the metabolism of the animal and also in an at least partly elimination of other microorganisms ingested with the food. The characteristic representatives of the intestinal flora in *Bibio* larvae belong to the families *Pseudomonadaceae* and *Enterobacteriaceae*. It may be supposed that the intestinal microflora shows a certain degree of qualitative and quantitative difference not only in the imagos and larvae of various insect species, but also in populations of the same species living in different environments. The alteration may not affect only the predominant bacteria but also those that survive the intestinal passage ("additional intestinal flora elements"). Fig. 1 shows bacterial species predominant in the intestine of larvae of larva-population No. 6 and in the environment. The sketch is a supplemented form of a scheme prepared on the basis of our previous zoological-bacteriological investigations [28]. The arrows indicate the direction of the material and energy flow. The predominant members of microflora and fauna developing in the litter composed mainly of the remainders of *Carpinus betulus*, *Quercus cerris* and Gramineae are as follows: *Amoeba terricola*, *Bodo ovatus*, *Colpoda cucullus*, *Difflugia globulus*, *Saprophilus muscorum*, *Actinomyces citreofluorescens*, *Streptomyces aureofaciens*, *Penicillium* spp., *Bacillus cereus* var. mycoides, *Bacillus subtilis*, *Mycobacterium* spp., *Agrobacterium* spp., etc. These organisms metabolize directly or indirectly the organic matters of the litter. They and other organisms contaminating the soil matter in the upper layer of horizon A_H are ingested by *Bibio* larvae. During intestinal passage the total bacterial count increases to manyfold, while the number of species decreases. In the digestive canal, where bacteria always gain an absolute predominance, actinomycetes are represented by *Streptomyces finlayi* and small numbers of *Actinomyces* (Streptomyces) *vulgaris*. The predominating bacterial species are *Ps. fluorescens*, *E. aerogenes*, intermediary *Enterobacteriaceae* strains, etc. Protozoa and rotifera [29] characteristic of the litter disappear completely. In the excrement actinomycetes (first of all *Str. finlayi* common also in the intestine) become predominant in a week after excretion and increase their number to 80% of the total flora. Certain bacteria

also multiply in the excrement to high numbers (*Azotobacter agilis*, *B. cereus* var. mycoides); yet, *Ps. fluorescens* still occurs very frequently. In a few days or weeks the microflora of the fresh excrement changes into a population characteristic of the "humified" excrement: many streptomycetes including the predominant *Str. chartreusis* and locally accumulating *Nocardia alba*, as well as a wide variety of bacteria, yeasts and protozoa. The scheme shown in Fig. 1 represents, of course, only one of the many possible combinations which may develop in different environments.

Further studies on the intestinal microflora of *Bibio* larvae will be presented in subsequent papers.

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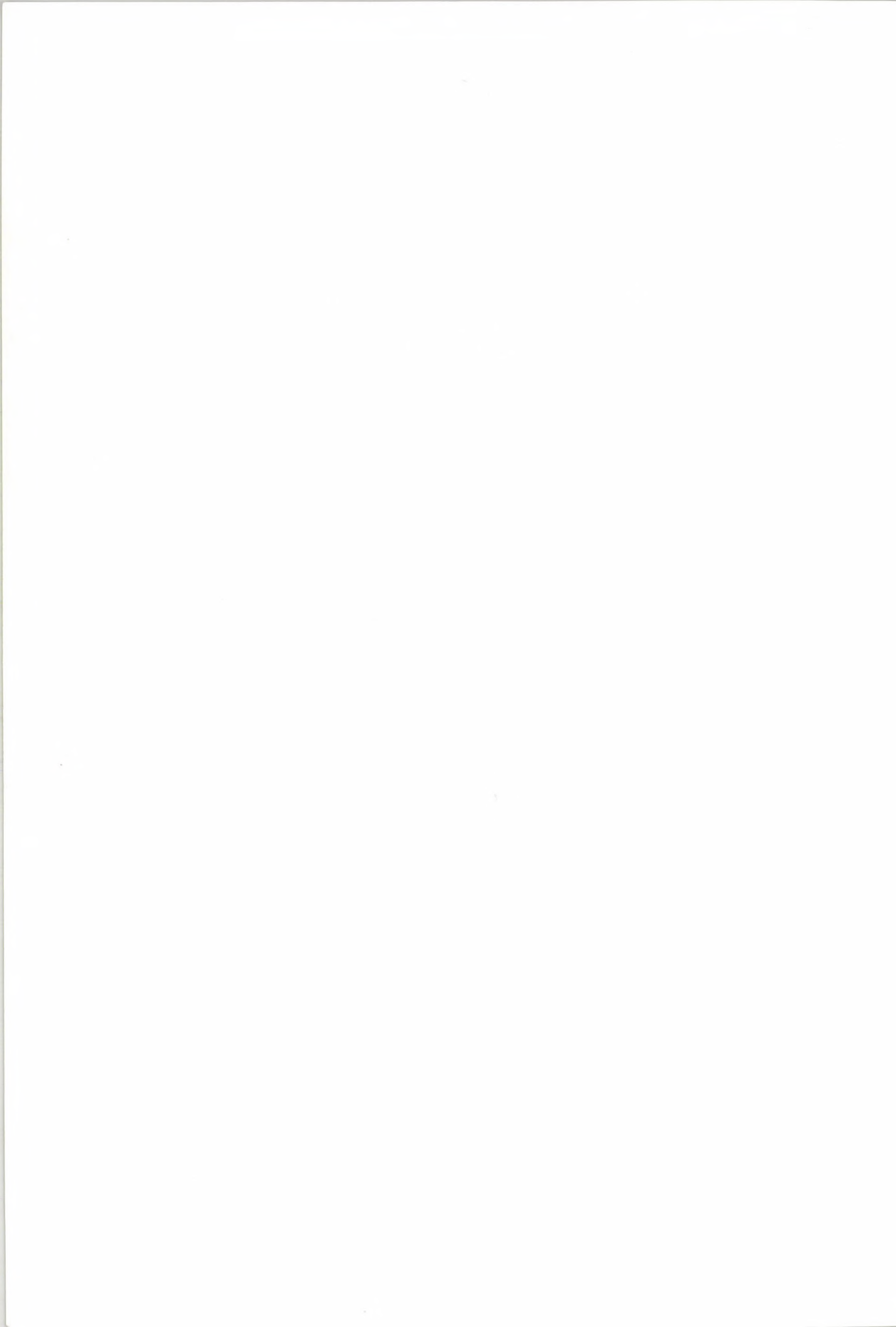


INDEX

Tomus XVI

Fasciculus 4

Ralovich, B., Vörös, S., Verténvi, A.: Antigenic Relationship between *Shigella sonnei* Phase II and *Enterobacter cloacae* 303

László, V. G., Kerekes, L.: Phage and Colicin Typing of *Shigella sonnei* 309

Tálas, M.: Interferon Stimulation and other Phenotypic Properties of Two Variants of Polyoma Virus 319

Lantos, J., Kiss, M., Lányi, B., Völgyesi, J.: Serological and Phage Typing of *Pseudomonas aeruginosa* Invading a Municipal Water Supply 333

Szentirmai, A., Horváth, I.: Properties of Acetohydroxyacid Synthetase in *Mycobacterium pellegrino* 337

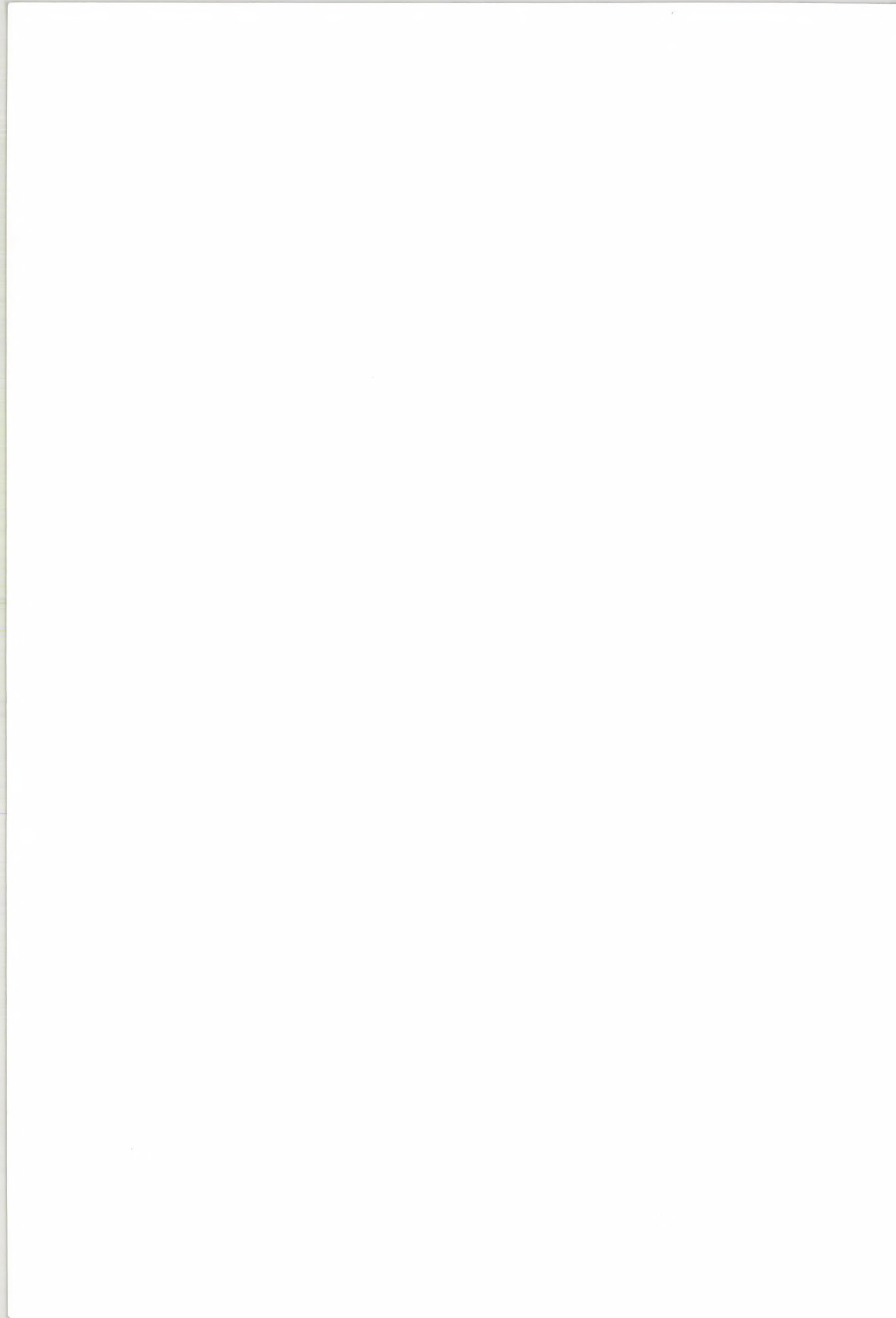
Horváth, I., Ott, I., Gadó, I.: Induction and Multiplication of λ-Phage. IV. The Effect of Panfuran 349

Lányi, B.: Amino Acid Utilization by Serologically Grouped *Pseudomonas aeruginosa* Strains 357

Szilágyi, T., Csernyánszky, H., Imre, S., Went, M.: The Effect of Hypothermia on Allergic-Type Skin Reactions 363

Weiszfeiler, J. G., Karasseva, V., Karczag, E.: Comparative Studies on the Taxonomic Relationship between *Mycobacterium abscessus* and *Mycobacterium borstelense.* Incidence of *Mycobacterium abscessus* in Monkeys 371

Szabó, I., Marton, M., Buti, I.: Intestinal Microflora of the Larvae of St. Mark's Fly. IV. Studies on the Intestinal Bacterial Flora of a Larva-population 381



SECOND INTERNATIONAL CONGRESS FOR VIROLOGY

Budapest, 5–11 September, 1971

FIRST CIRCULAR ANNOUNCING THE SECOND INTERNATIONAL CONGRESS FOR VIROLOGY

GENERAL POINTS. At a plenary session of the First International Congress for Virology the participants indicated warm approval for the proposal to set up a new Section of Virology within the IAMS. They also indicated a general wish to hold a second International Congress for Virology in 1971. Because the new Section of Virology could not be formed until the International Microbiology Meeting in 1970, they requested that the Convening Committee for the First Congress for Virology should begin making arrangements for the 1971 Congress and that responsibility for the arrangements could then be handed over to the new Virology Section in 1970.

We have made the following tentative arrangements:

LOCATION. The Congress will be held in a new hotel, Hotel DUNA Inter-Continental, situated on the embankment of the Danube in Budapest, Hungary. The hotel contains an auditorium for 1,000 persons and numerous smaller rooms for 50–200 persons each. A local organizing committee has been set up: Chairman, G. Ivánovics; Vice-Chairman, E. Farkas; Registrar, I. Dömök; and has begun to organize the local arrangements.

TIMING. The Congress will last for one week with five working days. The dates are September 5–11, 1971.

ELIGIBILITY FOR PARTICIPATION. In order to limit the participants to virologists, we propose once again to limit the membership to those who have contributed to some aspect of virology.

REGISTRATION FEE will be US $60.00 for full participants and US $25.00 for accompanying family members. The Registration Fee includes the cost of the Proceedings (International Virology II) which will be distributed to all participants. $50.00 should be sent to Account No. MNB – IBUSZ – RCS – 55.001, Budapest, Hungary, and $10.00 should be sent to the Secretary-General Dr. Melnick in Houston, U.S.A.

FORM OF THE CONGRESS. We have considered what principles should underlie the Congress. Plainly, the central theme must concern fundamental interpretations common to virology as a whole. At the same time, there are more restricted aspects bearing, for example, on epidemiology, plant pathology, particular groups of viruses, diagnostic problems and particular techniques.

Accordingly, we plan to hold five large symposia on topics of general interest common to all virologists. These will occupy the mornings. The afternoons will be occupied by several (at least six) simultaneous sessions of different aspects of virology of more restricted interest. Some of these can be strictly

small discussion groups composed of those working in the field (perhaps, 12—24 people) where the topic dictates it; others may be more heterogeneous and, accordingly, will have wider appeal (about 200 people).

We have suggested a number of topics for the programme (see below) which have been seen by a consultative board of 84 virologists working in 17 countries on all aspects of virology. We have received a good number of suggested modifications from these people, some of which we can incorporate when the programme is constructed.

TOPICS.

Large Symposia

Nucleic Acids — Structure — physical and chemical, Replication, Repair Mechanisms, Cell-free Systems

Replication of Viruses — Regulatory Mechanisms, Non-structural Proteins, Abortive Infections, Cell-free Systems

Viruses, Membranes, and Organelles — Role of organelles (nucleoli, polysomes, etc.) in the synthesis of viral components, including maturation at cell membranes

Structure and Function — This should include virus particles, structure and function of subviral components, viral assembly

Viruses Related to Cancer — Transformation, Persistence of Viral Genome, Immunological Events, Genetics

Specialized Topics

(One or more sessions can be arranged for specialized topics, if the wishes of the Congress participants are made known well in advance of the Congress)

Adenoviruses
Arboviruses
Bacterial Viruses
Cell-mediated Immunity in Virus Infections
Cooperative Infections
Defective Animal Viruses
Effect of Virus on Immunological Competence
Epidemiology
Formal Genetics
Herpesviruses
Host Components of the Virion and Their Role
Initiation of Infection
Insect-transmitted Plant Viruses
Interferon and Interference
Invertebrate Viruses
Lambda and Phage P22
Myxo- and Paramyxoviruses
Penetration of Viruses into Cells
Picornaviruses of Animals, Bacteria, Plants, Invertebrates
Plant Viruses
Poxviruses
Radiobiology of Viruses and Virus-infected Cell (X-ray, ultraviolet, and visible light)
Recent Advances in Human Virus Infections
Slow Viruses
Viral Chemotherapy — Mechanisms and Prospects
Virus-controlled Information Transfer
Virus Structure: Disassembly and Reassembly
Virus Taxonomy and Classification
Viruses, Antibodies, and Neutralization
Viruses of Algae and Fungi

LANGUAGE. As at the First International Congress for Virology, simultaneous interpretation will not be available. Even though all languages will be considered as official languages of the Congress, once again all speakers and discussants are requested to use English.

ADMINISTRATIVE DETAILS. In due course we shall distribute a Second Circular which will give further details. In the meantime we would like to have preliminary data on the expected number of participants and family members and on their needs for accommodation. For this purpose we attach a preliminary application form for participation. It should be returned by March 31, 1970 to:

Dr. I. Dömök, Registrar
2nd International Congress for Virology
MOTESZ
Apród u. 1,
Budapest I, Hungary

Further circulars will be sent to those who indicate their desire to participate in the Congress by returning this application.

Budapest, January, 1970.

CONVENING COMMITTEE

....

SECOND INTERNATIONAL CONGRESS FOR VIROLOGY
Budapest, September 5—11, 1971

Preliminary Application for Participation
(Please type or use block letters)

Family name .... First name(s) ....

Title or position ....

Institution ....

....

Mailing address ....

....

Presumably I will need .... single, double, Luxury,

1st Class, 2nd Class, Hotel, Private room/s/.

Please circle the desired accommodation.

Date ....

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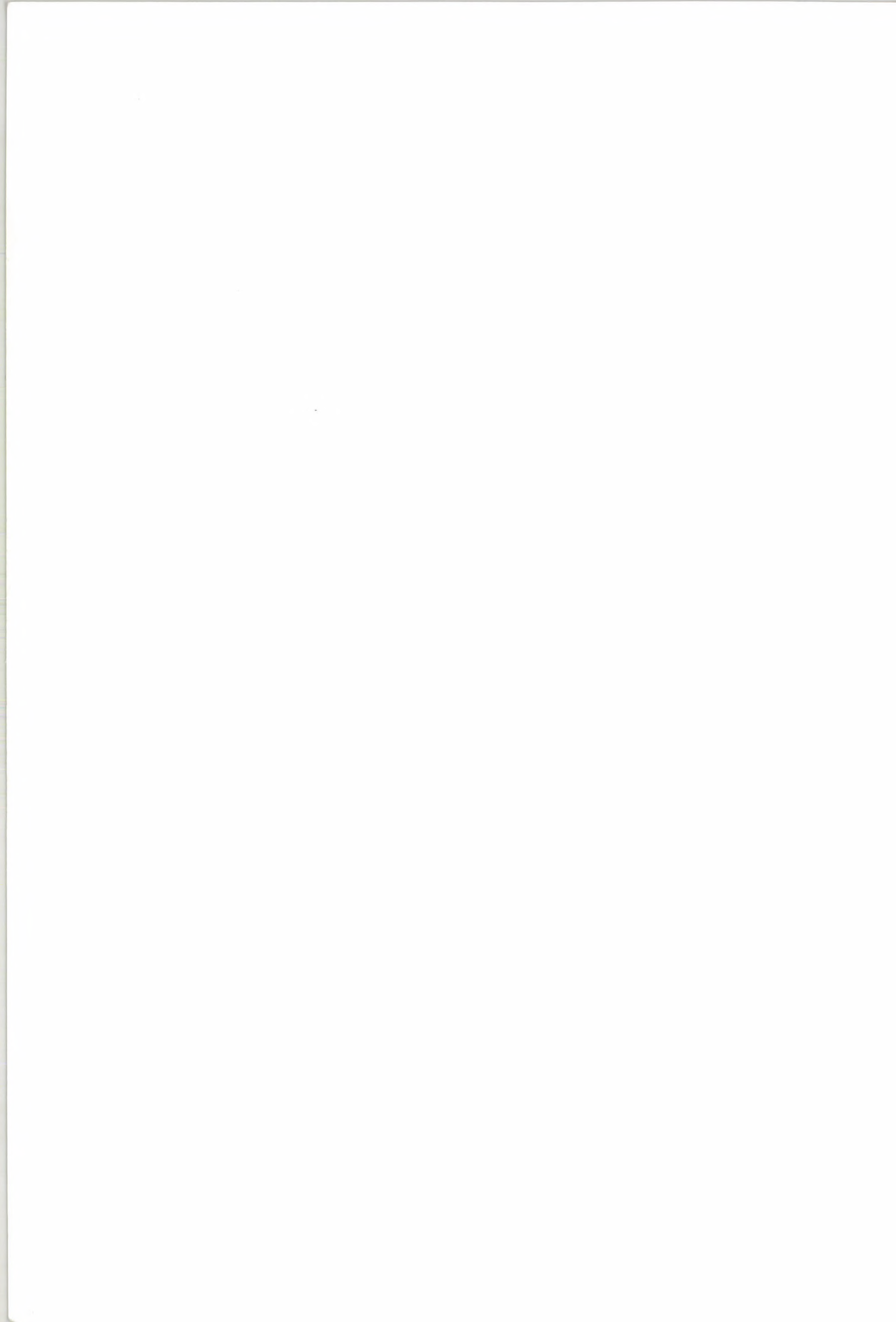

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ACTA MICROBIOLOGICA

ТОМ XVI

РЕЗЮМЕ

IMMUNOLOGICAL ACTIVITY OF RIBONUCLEOPROTEINS II. IMMUNE DIFFUSION STUDIES OF RIBONUCLEOPROTEINS EXTRACTED FROM GUINEA-PIG LIVER

K. MERÉTEY, V. VÁRTERÉSZ, E. ELEKES

ИММУНОЛОГИЧЕСКАЯ АКТИВНОСТЬ РИБОНУКЛЕОПРОТЕИНОВ II. ИССЛЕДОВАНИЕ ИММУННОЙ ДИФФУЗИЕЙ РИБОНУКЛЕОПРОТЕИНОВ, ЭКСТРАГИРОВАННЫХ ИЗ ПЕЧЕНИ МОРСКОЙ СВИНКИ

К. МЕРЕТЕИ, В. ВАРТЕРЕС, Е. ЭЛЕКЕШ

РНП-овая вытяжка из печени морской свинки и гомологичная иммунная сыворотка создают мультиплексную систему антиген-антитело. Число антигенных компонентов, выявляемых иммунэлектрофорезом, колеблется между 2 и 8. Некоторые из антигенов имеют нуклеопротеиновую природу. На это указывает антигенное свойство фракции нуклепротеинового характера, выделенной электрофорезом. Электросинеретические препараты антигена, меченного *in vivo* ^{32}P, и сыворотки дают положительную авторадиографическую картину. Выделенные фракции, содержащие только нуклеиновую кислоту и нуклеотиды, не играют роли в серологических реакциях. Антигенные компоненты оказались подобными антигенам, обнаруживаемым в субцеллюлярной фракции гомогенизата печени. Большая часть антигенов обладает видовой и органной специфичностью.

IMMUNOLOGICAL ACTIVITY OF RIBONUCLEOPROTEINS III. CHANGES IN MAMMALIAN RIBONUCLEOPROTEINS UPON THE EFFECT OF WHOLE BODY X-IRRADIATION

K. MERÉTEY, V. VÁRTERÉSZ, V. KUTAS, L. KOCSÁR

ИММУНОЛОГИЧЕСКАЯ АКТИВНОСТЬ РИБОНУКЛЕОПРОТЕИНОВ III. ИЗМЕНЕНИЯ В РИБОНУКЛЕОПРОТЕИНАХ МЛЕКОПИТАЮЩИХ НА ДЕЙСТВИЕ РЕНТГЕНОВОГО ОБЛУЧЕНИЯ ВСЕГО ТЕЛА

К. МЕРЕТЕИ, В. ВАРТЕРЕС, В. КУТАШ, Л. КОЧАР

В агар-электрофоретических препаратах РНП-овых экстрактов, выделенных из печени облученных и нормальных морских свинок, разделение нуклеиновой кислоты было различным. Изменения затронули, в основном, олигонуклеотидные фракции. По результатам иммунной диффузии и иммунэлектрофоретических исследований в антигенных компонентах вытяжки существенных изменений не произошло. Коэффициенты диффузии антигенных компонентов в случае нормальных вытяжек находились в пределах $0{,}5 \times 10^{-7}$ и $7{,}3 \times 10^{-7}$, в то время как в отношении облученных экстрактов они колебались между $0{,}7 - 9{,}9 \times 10^{-7}$. Нормальные и облученные РНП-овые экстракты в одинаковой степени обладали комплементсвязывающей активностью. В переваривании экстрактов РН-азой и трипсином не было отмечено никакой разницы. Облучение морских свинок, по-существу, не влияло на антигенность рибонуклеопротеинов печени.

PERSISTENCE OF HUMAN GAMMA-GLOBULIN IN ANIMALS

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ПЕРЗИСТЕНЦИЯ В ЖИВОТНЫХ ЧЕЛОВЕЧЕСКОГО γ-ГЛОБУЛИНА

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В организме морских свинок человеческий γ-глобулин не фрагментируется в течение 2—3 недель. После дачи человеческого γ-глобулина, меченного изотопом йода, наиболее высокую специфическую активность в течение 2 недель можно было обнаружить в крови, но высокие значения наблюдались также в печени, почках и лёгких. После введения человеческого γ-глобулина (0,125 г/кг веса тела) антитела, образуемые против него, появляются на второй неделе и остаются на высоком уровне ещё в течение 3 месяцев.

EFFECT OF DIBROMOMANNITOL ON DEXTRAN-INDUCED INCREASE OF LOCAL CAPILLARY PERMEABILITY AND ON ANAPHYLACTOID REACTION

G. LÁZÁR

ДЕЙСТВИЕ ДИБРОМОМАННИТОЛА НА УСИЛЕНИЕ ПРОНИЦАЕМОСТИ МЕСТНЫХ КАПИЛЛЯРОВ, ВЫЗВАННОЕ ДЕКСТРАНОМ, И НА АНАФИЛАКТОИДНУЮ РЕАКЦИЮ

Г. ЛАЗАР

Предварительная обработка дибромоманнитолом в большой степени подавляла усиление капиллярной проницаемости, вызванное методом **Halpern—Briot-a**, а также анафилактоидную реакцию крыс, обусловленную декстраном. Принимая во внимание, что в анафилактоидной реакции, вызванной декстраном у крыс, решающую роль играет выход гистамина и серотонина и упомянутым веществам присваивается роль также и в патогенезе местной реакции Шварцмана, на основе исследований автора может представиться вероятным, что горчичный азот и его производное, дибромоманнитол, обеспечивают подавление реакции Шварцмана не только посредством их действия, вызывающего лейкопению, но в подавлении играет роль и измененная готовность тканей реагировать.

BACTERIOLOGICAL MODEL EXPERIMENTS ON RADIOSTERILIZATION OF CATGUT

T. BARTHA, E. MÉRŐ, J. SZITA

БАКТЕРИОЛОГИЧЕСКИЕ МОДЕЛЬНЫЕ ОПЫТЫ В СВЯЗИ С РАДИОСТЕРИЛИЗАЦИЕЙ КЕТГУТА

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Авторы путем исследования естественного бактериального загрязнения проб кишечника овцы, а также на основе модельных опытов, в которых использовались пробы овечьего кишечника, зараженного аэробными споровыми штаммами бактерий, определили стерилизирующую дозу ионизирующего облучения.

Значение D_{10} бактериальных спор внутри кетгута было приблизительно в 2 раза больше такового спор, высушенных на поверхности фильтровальной бумаги. На основе результатов исследования для стерилизации кетгута предлагают дозу 3.5 ***Mrad***, при применении которой фактор достигает примерно 10^6, и эта величина с бактериологической точки зрения достаточная.

STUDIES ON PERITONEAL EXUDATE OF ANIMALS EXPERIMENTALLY INFECTED WITH T. GONDII. I. EFFECT OF HEAT ON ACTIVITY

M. AHMED SAMIR

ИССЛЕДОВАНИЕ ПЕРИТОНЕАЛЬНОГО ЭКСУДАТА ЖИВОТНЫХ, ЭКСПЕРИМЕНТАЛЬНО ЗАРАЖЕННЫХ TOXOPLASMA GONDII I. ВЛИЯНИЕ ТЕМПЕРАТУРЫ НА АКТИВНОСТЬ ЭКСУДАТА

М. АХМЕД САМИР

Введенный внутривенно супернатант перитонеального эксудата белых мышей, зараженных внутрибрюшинно ***Toxoplasma gondii***, вызывал конвульсии и гибель мышей. Токсический материал оказался термостабильным при 37° C и 50° C; с другой стороны, при воздействии на него 56° C-ной температуры в течение 30 минут наблюдался рост его активности (термопотенциация). Этот последний эффект можно было наблюдать также после разведения и высушивания материала. После 56° C-ной обработки возрасла опалесценция материала и изменилась его вискозность.

Повторное замораживание и оттаивание материала понижало токсичность, но не снимало её совсем. Фильтрат эксудатов (стеклянный фильтр с величиной пор 0,7—1,5 μ), разведенных физиологическим раствором, не оказался токсичным.

Автор пытается объяснить термопотенциацию и излагает предположения относительно природы материала.

REPLICATION OF VACCINIA VIRUS IN THE EMBRYONATED EGG IN THE PRESENCE OF METHISAZONE AN ELECTRON-MICROSCOPIC STUDY

B. LOVAS, I. HOLLÓS

РАЗМНОЖЕНИЕ ВИРУСА ВАКЦИНИИ В ЭМБРИОНИРОВАННОМ ЯЙЦЕ В ПРИСУТСТВИИ МЕТИСАЗОНА ЭЛЕКТРОННО-МИКРОСКОПИЧЕСКОЕ ИССЛЕДОВАНИЕ

Б. ЛОВАШ, И. ХОЛЛОШ

Авторы подавляли с помощью 20 *M* метисазона размножение вируса вакцинии в клетках хориоаллантоисной мембраны эмбрионированного яйца. За ходом размножения вируса следили титрованием инфективности и электронно-микроскопическим исследованием морфологии.

(1) Процессы, произошедшие в ранние этапы вирусного размножения до эклипса, оказались подобными таковым в контрольной (без подавления) системе.

(2) В первые 24 часа цикла в цитоплазме клеток молодые формы размножения не были найдены. Спустя 48 часов число появляющихся матрикс, производящих вирус, было незначительным, их структура зачастую была размытой, неправильной. Число незрелых вирусных форм, встречающихся в каждом скоплении было довольно низким, очертания частиц нередко были неправильными, их наружные мембраны имели недостатки в построении. Построение вироплазмы многих молодых частиц в отличие от большинства было аморфным, бесструктурным. Молодая форма, содержащая нуклеоид, встречалась редко. Такие срезы, в которых одновременно присутствовали бы зрелые и незрелые формы, найдены не были. Число зрелых вирионов было довольно низким.

(3) По ходу морфологических исследований авторы наблюдали два, до сих пор ещё не описанных явления. (а) Спустя 24 часа после заражения и обработки метисазоном встречались такие клетки, в цитоплазме которых в большой степени размножались расширенные ходы зернистого эндоплазматического **reticulum-a**. (б) Большую часть зрелого вириона окружала не только двойная элементарная мембрана, но и вакуоль.

EFFECT OF DIBROMOMANNITOL AND DIBROMODULCITOL ON IMMUNOLOGICAL AND MICROBIOLOGICAL SYSTEMS

G. ELEK, I. FÖLDES, E. VANDRA

ДЕЙСТВИЕ ДИБРОМОМАННИТОЛА И ДИБРОМОДУЛЬЦИТОЛА НА ИММУНОЛОГИЧЕСКИЕ И МИКРОБИОЛОГИЧЕСКИЕ СИСТЕМЫ

Г. ЕЛЕК, И. ФЁЛДЕШ, Е. ВАНДРА

Авторы изучали действие дибромодидеоксиманнитола (ДБМ) и его стереоизомера, дибромодидеоксидульцитола (ДБД), на иммунный ответ, рост *Mycobacterium phlei* и бляшкообразующую способность *Phagus phlei.* ДБД сильнее, чем ДБМ, подавлял иммунитет против столбняка у мышей и выработку антител против сывороточного белка у крыс. ДБМ не влиял на среднее время переживания гомойотрансплантатов кожи крыс, размноженных рандомизированно, ДБД увеличил это время на 2 дня. Несмотря на то, что из двух соединений меньшей растворимостью обладает ДБД, чем ДБМ, иммуносупрессивное действие первого было всё-таки больше, чем последнего.

Рост культур *Mycobacterium phlei* ДБД подавалял в более низкой концентрации, чем ДБМ. ДБД и ДБМ, добавленные к суспензии *Phagus phlei,* снижали титр фага. В противоположность предшествующим моделям ДБМ сильнее понижал число фагов, чем ДБД.

PRODUCTION OF ANTIBODIES AGAINST MYCOBACTERIUM PHLEI PHAGE

G. ELEK, E. VANDRA, I. FÖLDES

ВЫРАБОТКА АНТИТЕЛ MYCOBACTERIUM PHLEI ПРОТИВ ФАГОВ

Г. ЕЛЕК, Е. ВАНДРА, И. ФЁЛДЕШ

Авторы иммунизировали кроликов **phagus phlei**, затем изучали *in vitro* фагнейрализующее действие клеток селезенки этих кроликов частью подсчётом фагов, частью изотопным методом (с применением фагов **phlei**, мечены ^{32}P). Число фагов **phlei**, инкубированных в растворе Паркер вместе с клетками селезенки иммунизированных животных, в бесклеточном супернатанте, полученном после центрифугирования, в течение 4-часового эксперимента постепенно понизилось. Актиномицин-С или большая доза хлорамфеникола спустя 30 минут снимали фагнейтрализующее действие иммунных клеток селезенки. И фагнейтрализующее действие иммунных клеток селезенки, обработанных ультразвуком, температурой и противолимфоцитарной сывороткой, продолжалось также только 30 минут. Если иммунные клетки селезенки предварительно инкубировались с фагом **phlei**, то в среде, содержащей иммунные клетки селезенки, выявлялись антитела, несвязанные с клеткой.

TRANSFORMATION OF PHAGE RESISTANCE

S. HORVÁTH

ТРАНСФОРМАЦИЯ ФАГОВОЙ РЕЗИСТЕНТНОСТИ

И. ХОРВАТ

Штамм *B. subtilis* 168 M **try**$^-$ **phs** — является клеткой хозяина фага SPO—1. Автор из клеток этого штамма выделил спонтанный мутант 168 M **try**$^-$ **phr** (фагрезистентный). Мутационная пропорция оказалась 8.32×10^{-6}. В течение опытов по трансформации автор селектировал фагрезистентные клетки как **try**$^+$ **phr**, трансформанты. Кривые компетенции, начерченные в отношении формирования во времени числа трансформантов **try**$^+$ **phs**, а также **try**$^+$ **phr**, были примерно одинаковыми. Число трансформантов **try**$^+$ **phs**, а также **try**$^+$ **phr** зависело от концентрации ДНК. Характеристики **try**$^+$ и **phr** не оказались связанными.

В течение трансформации число трансформантов **try**$^+$ **phs** быстро поднялось до высокого значения с 5-и часовой фенотипической **lag** фазой. Трансформанты **try**$^+$ **phr** появились после 6,5-часовой инкубации.

Частота трансформантов **try**$^+$ **phr** в сравнении с трансформантами **try**$^+$ **phs** была 0,12—0,032%.

BIOCHEMICAL STUDIES ON STREPTOMYCES AUREOFACIENS V. THE ROLE OF COBALAMINS AND METHIONINE IN METHYLATION REACTIONS IN TETRACYCLINE BIOSYNTHESIS

M. JÁRAI

БИОХИМИЧЕСКОЕ ИЗУЧЕНИЕ STREPTOMYCES AUREOFACIENS V. РОЛЬ КОБАЛАМИНОВ И МЕТИОНИНА В РЕАКЦИИ МЕТИЛИРОВАНИЯ БИОСИНТЕЗА ТЕТРАЦИКЛИНА

М. ЙАРАИ

Автор изучал роль кобаламинов в биосинтезе 7-хлортетрациклина в случае *S. aureofaciens* в отношении инкорпорации С—6-метила. Было выявлено, что у штамма *S. aureofaciens* С—13, который приблизительно в одинаковой пропорции производит хлортетрациклин и диметилхлортетрациклин, на питательной среде, содержащей кобаламин, прекращается производство диметилхлортетрациклина. Ион кобальта вместе с другими ионами — оказался неэффективным, тогда как с производным кобаминовой кислоты, обладающей антагонистическим действием на штамм *E. coli* 113—3, можно было понизить активность метилирования.

На штамме *S. aureofaciens* С—13 изучалось действие метионина — а также метионин-метил—C^{14} — на синтез группы 6-метил тетрациклиновой цепи. Активность метилирования можно было также восстановить метионином — в высокой концентрации, — добавленным к ферментации.

Автор выявил, что добавляя метионин, цианокобаламин, аденин в высокой концентрации в питательную среду, используемую для приготовления вакцин, а затем после отмывки, прививая полученный мицелий, также можно понизить активность метилирования во время ферментации.

COMPARATIVE STUDIES ON THE BIOLOGICAL PROPERTIES OF ESCHERICHIA COLI O124:K72(B17) STRAINS

S. DEÁK, M. ÁDÁM

СРАВНИТЕЛЬНОЕ ИССЛЕДОВАНИЕ РАЗЛИЧНЫХ БИОЛОГИЧЕСКИХ СВОЙСТВ ШТАММОВ ESCHERICHIA COLI O124:K72(B17)

Ш. ДЕАК, М. АДАМ

В случае 725 штаммов *E. coli* O124 авторы исследовали лизогенные, колициногенные свойства 725 штаммов, их жгутиковый антиген и время разложения лактозы- В результате исследований была разработана схема группировки штаммов, типированных на основе свойств фага и колицина. По лизогенным, колициногенным свойствам штаммы были подразделены на 4 группы и внутри каждой из них—на 7 типов. Авторы установили, что колициногенные штаммы встречаются в большем проценте, чем лизогенные штаммы. В исследуемом материале наибольшим числом был представлен колициногенный штамм 1 типа. Лизогенные штаммы 2 типа в отличие от других штаммов располагали более коротким временем лактозной ферментации, и все штаммы, относящиеся в этот тип, оказались безжгутиковыми. Из жгутиковых штаммов в наибольшем количестве встречались штаммы, располагающие антигеном H30; внутри 4 типа был найден в небольшом количестве штамм со жгутиковым антигеном H27, тогда как в случае 4 штаммов, входящих в этот же тип, известный H-антиген не выявлялся.

Для дифференцирования колицинов и фагов, происходящих из штаммов *E. coli* O124, исследовалась чувствительность веществ, подавляющих рост бактерий, к температуре, хлороформу, концентрации ионов H^+ и трипсину.

В случае штаммов типа 1 в, обладающих в одинаковой степени лизогенными и колициногенными свойствами, посредством лизогении удалось подтвердить способ возникновения штаммов среди естественных условий.

STUDIES ON SWINE SERUM IMMUNOGLOBULINS
I. PROTEIN FRACTIONS IN SWINE SERUM

J. SURJÁN

ИЗУЧЕНИЕ ИММУНГЛОБУЛИНОВ СЫВОРОТОК СВИНЕЙ
I. БЕЛКОВЫЕ ФРАКЦИИ СЫВОРОТКИ СВИНЬИ

Й. ШУРЬЯН

Изучение состава белка свиной сыворотки проводилось такими кроличьими сыворотками, которые в пробирочном опыте преципитировали сыворотку свиньи даже в разведениях 10^{-6}, 10^{-7}. Суммируя результаты, полученные с различными кроличьими сыворотками, с помощью иммунэлектрофореза удалось выделить из сыворотки свиньи 28 составных частей. Распределение фракций: альбумин, 3 преальбумин, $7\alpha_1$-глобулин, $5\alpha_2$-глобулин, $5\beta_1$-глобулин, $4\ (+1)\beta 2$-глобулин, 2γ-глобулин. Один из β_1-глобулинов, по всей вероятности, подобен трансферину. Среди глобулиновых фракций хорошо распознаются 3 наиболее важных иммунглобулина, **IgG**, **IgA** и **IgM**.

EFFECT ON INFLUENZA VIRUS OF A MODIFIED FRANCIS INHIBITOR AND ITS ACETONE-SOLUBLE FRACTION
III. EXPERIMENTS ON THE MOUSE LUNG

I. HOLLÓS

ДЕЙСТВИЕ МОДИФИЦИРОВАННОГО FRANCIS-ИНГИБИТОРА И ЕГО АЦЕТОНОРАСТВОРИМОЙ ФРАКЦИИ НА ВИРУС ГРИППА
III. ОПЫТЫ НА ЛЕГКИХ МЫШЕЙ

И. ХОЛЛОШ

На легких мышей изучалось подавляющее действие **Francis**-ингибитора (K_0), солевого производного диэтил-р-фенилен-диазониума (*8m*) и ацетонорастворимой фракции последнего (*8ac*) на размножение вируса гриппа А—1. Вещества вводились внутрибрюшинно. Значительное подавляющее действие было оказано у *8m* против 50 $ИД_{50}$ и у *8ac* против 800 $ИД_{50}$ вируса. При большей вирусной дозе *8ac* оказывала подавляющий эффект только в количестве с токсическим действием. Вдыхание растворимой в эфире фракции *8ac* подавляло размножение вируса даже при дозе последнего 5×10^5 $ИД_{50}$.

Автор определил некоторые химические и физикохимические свойства упомянутых выше веществ. Найдено, что содержание нейраминовой кислоты в веществах и её распад под действием вирусной нейраминидазы являются характерными.

INDUCTION AND MULTIPLICATION OF λ-PHAGE
III. THE EFFECT OF HYDROXYUREA ON HEAT INDUCTION

I. GADÓ, É. BOROMISSZA, I. HORVÁTH

ИНДУКЦИЯ И РАЗМНОЖЕНИЕ λ-ФАГА
III. ВЛИЯНИЕ HYDROXYUREA НА ТЕМПЕРАТУРНУЮ ИНДУКЦИЮ

И. ГАДО, Е. БОРОМИССА, И. ХОРВАТ

Hydroxyurea, обладающая противораковым действием и подавляющая синтез ДНК, не индуцирует, подобно митомицину и ультрафиолетовому облучению, штамм *E. coli* Е—12 (λ cl—857), индуцируемый температурой. Эффект **hydroxyurea** на температурную индукцию профага зависит от условий. На полной питательной среде в небольшой степени замедляет депрессию профага, возникающую на действие температуры. В состоянии „**shift down**“ чувствительность процесса к **hydroxyurea** намного выразительнее.

BREAKDOWN OF OLIGOSACCHARIDES BY SOME AFERMENTATIVE YEAST SPECIES

E. K. NOVÁK, E. DARVAS, J. ZSOLT

РАСЩЕПЛЕНИЕ ОЛИГОСАХАРИДОВ НЕКОТОРЫМИ АФЕРМЕНТАТИВНЫМИ ВИДАМИ ДРОЖЖЕЙ

Е. К. НОВАК, Е. ДАРВАШ, Й. ЖОЛТ

Изучалось расщепление мальтозы, сахарозы и раффинозы четырьмя аферментативными дрожжами (***Bullera tsugae, Sporobolomyces singularis, Debaryomyces vini*** и ***Rhodotorula zsoltii***) в случае полных и обработанных ацетоном клеток, а также в случае экстрактов, свободных от клеток. У всех четырех видов наблюдали ацетоночувствительное расщепление мальтозы. Подобные предполагаемым энзимам встречаются и у большинства других дрожжей. Внеклеточный энзим ***Rhodotorula zsoltii***, расщепляющий сахарозу, пока только сомнительно может быть принят за инвертазу.

EFFECT OF ENDOTOXIN ON THE SERUM LEVEL OF COMPLEMENT COMPONENTS
II. EFFECT OF ENDOTOXIN ON DOG SERUM COMPLEMENT LEVEL IN VIVO AND IN VITRO

G. FÜST, M. KERESZTES

ДЕЙСТВИЕ ЭНДОТОКСИНА НА УРОВЕНЬ КОМПОНЕНТОВ СЫВОРОТОЧНОГО КОМПЛЕМЕНТА
II. ИЗМЕНЕНИЕ ТИТРА КОМПЛЕМЕНТА В СЫВОРОТКЕ СОБАКИ НА ДЕЙСТВИЕ ЭНДОТОКСИНА IN VIVO И IN VITRO

Г. ФЮШТ, М. КЕРЕСТЕШ

Авторы изучали ***in vivo*** и ***in vitro*** изменение титра комплемента и его компонентов в сыворотке собаки на действие эндотоксина ***Serratia marcescens***, а также занимались «естественными» антителами, играющими роль в комплементпонижающем действии эндотоксина.

Было установлено, что после внутривенного введения сублетальной дозы эндотоксина, а также в процессе совместной **in vitro** инкубации сыворотки собаки и эндотоксина общий титр комплемента и титры четырех «классических» факторов комплемента значительно понижаются.

Авторы не нашли взаимосвязи между титром антител к эндотоксину и степенью понижения уровня комплемента ни в случае исследования индивидуальных человеческих сывороток, ни при сравнительном исследовании сывороток разных видов животных. Понижение титра комплемента имело место даже и тогда, когда антитела из сыворотки полностью удалялись.

На основе опытов и сопоставления с литературными данными представляется вероятным, что комплемент-система, активированная эндотоксином, играет роль в патомеханизме эндотоксинного шока. Наблюдаемое понижение уровня комплемента ***in vivo*** и ***in vitro*** возникает не как или не в полной мере как результат реакции антиген-антитело.

INVESTIGATIONS ON THE OLIGOSACCHARIDE DECOMPOSITION BY ENDOMYCOPSIS WICKERHAMII VAN DER WALT

E. K. NOVÁK, F. KEVEI, B. OLÁH, J. ZSOLT

ИЗУЧЕНИЕ РАЗЛОЖЕНИЯ ОЛИГОСАХАРИДА ENDOMYCOPSIS WICKERHAMII VAN DER WALT

Е. К. НОВАК, Ф. КЕВЕИ, Б. ОЛАХ, Й. ЖОЛТ

В процессе изучения разложения олигосахарида ***Endomycopsis wickerhamii*** не удалось выделить энзим, разлагающий раффинозу. Энзим, разлагающий сахарозу, не относится к типу инвертазы, а похож на тот, который был выделен из других дрожжевых грибов. Энзим, разлагающий мальтозу, отличается от того, что было найдено у других дрожжей, так как оказался чувствительным к ацетону.

OBSERVATIONS ON PRIMARY BACTERIAEMIA

B. RALOVICH, L. EMŐDY

НАБЛЮДЕНИЯ, КАСАЮЩИЕСЯ ПЕРВИЧНОЙ БАКТЕРИЕМИИ

Б. РАЛОВИЧ, Л. ЕМЁДИ

Авторы заражали перорально мышей вирулентным для последних штаммом *S. enteritidis* и апатогенным штаммом *E. coli* O111 : B4 : H2. На основе полученных результатов считают, что первичная бактериемия, наблюдавшаяся при заражениях, проведенных *per os* не в естественной форме, не является следствием активного поступления бактерий из кишечного тракта в кровяное русло, а сопровождает метод проведения заражения.

NEW OBSERVATIONS ON ORAL VACCINATION AGAINST DYSENTERY

K. RAUSS, I. KÉTYI, J. MÁTÉ, P. KNEFFEL, G. AMBÓ, J. MARÓCZI, S. PUSZTAI, I. JÓÓ, L. SZENDREI

ДАЛЬНЕЙШИЕ НАБЛЮДЕНИЯ ОТНОСИТЕЛЬНО ИММУНИТЕТА ПРОТИВ ДИЗЕНТЕРИИ ПОСЛЕ ПЕРОРАЛЬНОЙ ВАКЦИНАЦИИ

К. РАУШШ, И. КЕТЬИ, Й. МАТЕ, П. КНЕФФЕЛ, Г. АМБО, Й. МАРОЦИ, Ш. ПУСТАИ, И. ЙОО, Л. СЕНДРЕИ

(1) Высокий уровень антител (защищающий мышей от заболевания) у взрослых, иммунизированных ранее парэнтерально адсорбированной дизентерийной вакциной, мог быть продлен в течение 6 месяцев эксперимента ревакцинацией через рот, если каждую неделю или 1 раз в две недели применяли 5-кратное количество антигена по сравнению с парэнтеральной дозой (приблизительно 0,5 мг сухого антигена **Boivin** на каждый компонент). С точки зрения поддержания иммунитета оральная ревакцинация, проведенная с тем же количеством антигена, но при интервалах 3 и 4 недель, оказалась безрезультатной.

(2) Дети, иммунизированные адсорбированной вакциной — независимо от того, два или 1 раз они прививались, — на ревакцинацию реагировали подобно взрослым.

(3) Исходя из наблюдений, для практики можно сделать вывод, что ревакцинации, принимая во внимание необходимую осторожность, нужно проводить с интервалом 10 дней.

(4) У взрослых парэнтеральная иммунизирующая доза с 30-кратным количеством (приблизительно 3,0 мг сухого антигена **Boivin**), введенным в два приема через рот, может обеспечить — в отражении антител, защищающих мышей, — надежный иммунитет против дизентерии.

(5) У детей оральная основная иммунизация, проведенная дозами, примененными у взрослых, или более высокими, только в меньшей степени повысила уровень титра, защищающего мышей, но после ревакцинации наблюдался желаемый подъем титра. Объяснение может быть найдено в продленном антигенном раздражении.

(6) В сыворотке орально ревакцинированных независимо от того, парэнтерально или орально была проведена основная иммунизация, после окончания курса вакцинации высокий титр антител сохранялся, по крайней мере, в течение 2 месяцев, что опять обращает внимание на значение продленных небольших антигенных стимулов.

(7) Авторы указали на преимущество антигена **Boivin** перед корпускулярными вакцинами.

SELECTIVE IN VITRO ANTIVIRAL EFFECT ON MYXOVIRUS STRAINS OF GUANIDINE DERIVATIVES

L. VÁCZI, O. HANKOVSZKY, K. HIDEG, L. GERGELY, GY. HADHÁZY

СЕЛЕКТИВНОЕ АНТИВИРУСНОЕ ДЕЙСТВИЕ ПРОИЗВОДНЫХ ГУАНИДИНА НА МИКСОВИРУСНЫЕ ШТАММЫ IN VITRO

Л. ВАЦИ, О. ХАНКОВСКИ, К. ХИДЕГ, Л. ГЕРГЕЙ, ДЬ. ХАДХАЗИ

Авторы изучали **in vitro**, на переживающих кусочках хориоаллантоисной мембраны, действие гуанидина и 16 его производных, подавляющее размножение штаммов гриппа A—O, PR8 и парагриппа —1, Сендай. Была найдена взаимосвязь определенной степени между химической структурой соединений и их эффективностью.

DISTRIBUTION IN THE ORGANS OF 131I-OVALBUMIN ANTIGEN IN RAT AND MOUSE ANAPHYLAXIS

B. CSABA, G. DESEŐ, M. KÁVAI

РАСПРЕДЕЛЕНИЕ АНТИГЕНА 131J-ОВАЛЬБУМИНА В ОРГАНАХ КРЫС И МЫШЕЙ ПРИ АНАФИЛАКТИЧЕСКОМ ШОКЕ

Б. ЧАБА, Г. ДЕШЕЁ, М. КАВАИ

(1) У крыс при анафилактическом шоке — на основе выявления распределения антигена — большое количество 131J-овальбумина (ОА) фиксируется в тонком кишечнике, легких и сердце; предполагается, что некоторая часть его специфически связывается с антителами, имеющимися в этих органах. На основе этого авторы предполагают, что наряду с тонким кишечником в патомеханизме анафилактического шока крыс также играют роль легкие и сердце.

(2) У мышей при анафилактическом шоке наибольшая активность 131J—ОА может быть измерена в легких, вместе с тем, однако, активность антигена тонкого кишечника, а также кожи значительно выше, чем в контроле. Таким образом, в патомеханизме анафилактического шока мышей значительную роль можно отвести, в первую очередь, легким.

(3) На основе полученных результатов авторы искали и нашли взаимосвязь между симптомами анафилаксии у крыс и мышей и локализацией антител в органах. По их мнению все симптомы анафилаксии могут быть хорошо объяснены выпадением функции органов, пораженных и органически за счёт реакции антиген-антитело.

SOME PROPERTIES OF ALKALINE PHOSPHATASE IN BACILLUS SPECIES

A. DOBOZY, H. HAMMER

ИЗУЧЕНИЕ НЕКОТОРЫХ СВОЙСТВ ЩЕЛОЧНОЙ ФОСФАТАЗЫ В BACILLUS SPECIES

А. ДОБОЗИ, Х. ХАММЕР

Щелочная фосфатаза *B. anthracis*, *B. cereus*, *B. megaterium* и *B. subtilis* оказалась репрессибельным энзимом. Из фосфатаз четырех штаммов энзим *B. subtilis* репрессировался при самом низком, в то время как энзим *B. cereus* при самом высоком содержании фосфата в питательной среде.

Специфичность к субстратам четырех штаммов, исследовав гидролиз **para- Nitrophenylfosfat-a**, **β-Glicerofosfat-a**, адениловой кислоты, **Glucose-1-fosfat-a**, **Glucose-6-fosfat-a** и **Pyrofosfat-a**, показывала только небольшую разницу.

Постоянная **Michaelis** изученных штаммов находится между $2{,}8\times10^{-5}$ и $4{,}1\times\times10^{-5}M$.

Чувствительность фосфатазы штаммов **Bacillus** в отношении комплекс-образователей и температары в случае каждого штамма показывала характерную разницу.

HEART GLYCOSIDES IN POLIOVIRUS HOST CELL INTERACTION I. EFFECT OF DIGOXIN AND DIGITOXIN AND THEIR AGLUCONS ON ONE STEP GROWTH CURVES

A. KOCH, E. GYÖRGY

ВЛИЯНИЕ СЕРДЕЧНЫХ ГЛЮКОЗИДОВ НА ВЗАИМОДЕЙСТВИЕ ПОЛИОВИРУСА И КЛЕТКИ I. ДЕЙСТВИЕ ДИГОКСИНА, ДИГИТОКСИНА И ИХ АГЛЮКОНОВ НА ОДНОСТУПЕНЧАТУЮ КРИВУЮ РАЗМНОЖЕНИЯ

А. КОХ, Е. ДЬЁРДЬ

В системе, состоящей из штамма Махони полиовируса 1 типа и клеток перманентной клеточной линии почки обезьяны РМК III/1, в питательной среде Хэнкса с глюкозой наблюдали понижение количества образующегося вируса и вытягивание lag-фазы в при-

сутствии 10^{-6} или 10^{-7} M/l дигитоксина, дигоксина и соответствующих аглюконов. Оба глюкозида повышали количество образующихся вирионов и сокращали lag-фазу в зависимости от концентрации, в зоне последней 10^{-8} и 10^{-11} M/l. Два максимальных и минимальных подавления наблюдались при концентрациях 10^{-6} и 10^{-8} M/l, а также 10^{-7} и 10^{-11} M/l. Подавляющее действие дигитоксигенина до концентрации 10^{-6}—10^{-8} M/l имело тенденцию к понижению, при концентрации 10^{-9} M/l и менее никакого эффекта не наблюдалось.

HEART GLYCOSIDES IN POLIOVIRUS HOST CELL INTERACTION II. EFFECT OF THE TIME OF ADDITION ON STIMULATORY OR INHIBITORY ACTION

E. GYÖRGY, A. KOCH

ВЛИЯНИЕ СЕРДЕЧНЫХ ГЛЮКОЗИДОВ НА ВЗАИМОДЕЙСТВИЕ ПОЛИОВИРУСА И КЛЕТКИ II ВЛИЯНИЕ ВРЕМЕНИ ДОЗИРОВКИ НА УСИЛИВАЮЩУЮ ИЛИ ПОДАВЛЯЮЩУЮ АКТИВНОСТЬ

Е. ДЬЁРДЬ, А. КОХ

После адсорбции штамма Махони полиовируса 1 типа к суспензии клеток РМК III/1 в различное время были добавлены дигитоксин, дигоксин и их аглюконы в концентрации 10^{-6}, 10^{-8} и 10^{-9} M/l. Эффект измерялся начертанием кривой одноступенчатого роста. Время прибавления значительно влияло на эффективность как подавления, так и усиления. В каждом случае максимальный эффект давало прибавление в 0 время. При низкой концентрации добавление через час или позднее уже не оказывало эффекта.

При высоких концентрациях подавляющее действие было тем меньше, чем позже происходило добавление. В случае дигитоксигенина, дигоксина и дигоксигенина резкое падение эффекта наблюдалось между первым и вторым часом. Кажется, что производные дигиталиса подавляют необратимую связь и/или ранний период принятия вириона (эклипс).

INHIBITION OF THE DEVELOPMENT OF PRIMARY ANTITOXIC IMMUNITY BY HETEROLOGOUS ANTILYMPHOCYTE SERUM

L. RÉTHY, L. HEGEDÜS

ПОДАВЛЕНИЕ ОБРАЗОВАНИЯ АНТИТОКСИЧЕСКОГО ИММУНИТЕТА ГЕТЕРОЛОГИЧНОЙ АНТИЛИМФОЦИТАРНОЙ СЫВОРОТКОЙ

Л. РЕТИ, Л. ХЕГЕДЮШ

Авторы изучали на мышах, иммунизированных градуированными дозами столбнячного анатоксина и подвергнутых исследованию на токсинную толерантность, действие гетерологичной антилимфоцитарной сыворотки в отношении образования первичного иммунитета против активного столбняка. По ходу опытов было установлено следующее:

1. Гетерологичная антилимфоцитарная сыворотка (АЛС) эффективно подавляла образование первичного иммунитета против столбняка.
2. Иммуносупрессивная обработка, проведенная АЛС, оказалась эффективной в ранней стадии первичной иммунизации. Авторы по ходу опытов не нашли разницы в действии АЛС независимо от того, проводилась ли иммуносупрессия непосредственно перед введением иммунизирующего антигена или сразу же после него.
3. АЛС не оказывала иммуносупрессивного действия в продуктивной фазе антитоксического иммунитета.
4. Из теорий, объясняющих механизм действия АЛС, полученными результатами подтверждаются теории, связанные с явлением «Blindfolding».
5. При иммуносупрессивном действии АЛС — в экспирементальных условиях— удалось исключить возможность «антигенной конкуренции».

THE EFFECT OF BORDETELLA PERTUSSIS VACCINE ON THE HISTAMINE METABOLISM OF RAT TISSUES

B. CSABA, L. MUSZBEK, L. KASSAI

ЭФФЕКТ ВАКЦИНЫ BORDETELLA PERTUSSIS НА ГИСТАМИНОВЫЙ МЕТАБОЛИЗМ ТКАНЕЙ КРЫСЫ

Б. ЧАБА, Л. МУСБЕК, Л. КАШШАИ

В ответ на действие вакцины *Bordetella pertussis* (БПВ) поднялся уровень гистамина в тканях крыс и увеличилось содержание свободного гистамина в моче. Параллельно с этими изменениями понижается способность тканей разлагать гистамин. Максимум изменений падает на 4 день после введения вакцины. Активность гистидиндекарбоксилазы существенно повысилась только в пилорической части желудка.

Подъем уровня гистамина относят, в первую очередь, за счёт пониженной способности тканей разлагать гистамин. У животных с удаленными надпочечниками БПВ не оказывала эффекта, вызвала повышение уровня гистамина или понижение гистаминразлагающей способности тканей. Лечение картизоном прекращало или значительно понижало эффект такого характера коклюшной вакцины. Полученные результаты доказывают, что БПВ в первую очередь с вызыванием недостаточности функции надпочечников непрямым путем оказывает действие на тканевой метаболизм гистамина.

FATTY ACIDS IN STAPHYLOCOCCUS AUREUS AND ESCHERICHIA COLI CULTURED IN VIVO

I. RÉDAI, A. RÉTHY, F. ROZGONYI

ИССЛЕДОВАНИЕ ЖИРНЫХ КИСЛОТ STAPHYLOCOCCUS AUREUS И ESCHERICHIA COLI, КУЛЬТИВИРОВАННЫХ IN VIVO

И. РЕДАИ, А. РЕТИ, Ф. РОЗГОНЬИ

1. Распределение клеток эксудата брюшной полости морской свинки зависит от вызывающего раздрожителя. После введения 1% туши 94—96% клеток составляли сегментированные лейкоциты, 4—6%-лимфициты. В эксудате, образовавшемся под влиянием 4% декстрана, пропорция сегментированных лейкоцитов составляла 70—80%, палочкоядерных лейкоцитов — 14—24%, лимфоцитов — 4—8%. На введение 10% казеина в эксудате, кроме указанных выше клеток, в 8—10% встречались клетки, по всей вероятности, эпителиального происхождения.

2. Распределение всех жирных кислот в клетках, находимых в брюшно-полостном эксудате морской свинки: жирные кислоты с C_{12} и более короткой углеродной цепочкой — 21—66%, пальмитиновая кислота — 10—23%, стеариновая кислота — 9—28% и насыщенные жирные кислоты — 8—19%.

3. В случае ***Staphylococcus aureus***, культивированного in vivo, 23—42% от общего содержания жирных кислот составляют ненасыщенные жирные кислоты. Распределение основных жирных кислот: антеизо—C_{15}—10—25%, пальмитиновая кислота — 12—19%, стеариновая кислота — 16—28%, масляная кислота — 12—22%, линоленовая кислота — 6,1—14% и арахиновая кислота — 3—11%.

4. В спектре жирных кислот ***E. coli***, культивированных in vivo, могут быть выявлены только следы жирной кислоты, содержащей циклопропановое кольцо. Наиболее основные компоненты спектра: пальмитиновая кислота — 24,8%, стеариновая кислота — 22,2%, масляная кислота — 15,7% и линоленовая кислота — 12,4%.

EFFECT OF EMETINE ON THE MULTIPLICATION OF PSEUDORABIES AND SEMLIKI FOREST VIRUSES AND ON THE INTERFERON PRODUCTION BY CULTURED CELLS

I. ROSZTÓCZY

ЭФФЕКТ ЭМЕТИНА НА РАЗМНОЖЕНИЕ ВИРУСОВ ПСЕВДОБЕШЕНСТВА И ЛЕСА СЕМЛИКИ, А ТАКЖЕ НА ПРОДУКЦИЮ ИНТЕРФЕРОНА КЛЕТКАМИ ТКАНЕВОЙ КУЛЬТУРЫ

И. РОСТОЦИ

Эметин и 2-дегидроэметин подавляли размножение вирусов псевдобешенства и Леса Семлики только в концентрации, подавляющей деление фибробластов куриного эмбриона. Наиболее чувствительным к упомянутым веществам оказался начальный период цикла размножения примененных вирусов. Синтез интерферона, индуцированного аденовирусом 8 типа в фибробластах куриного эмбриона, подавлялся даже 10-кратным разведением концентрации, подавляющей размножение вируса. В низкой концентрации эметин понижал действие интерферона. Эметин и 2-дегидроэметин не влияли на переживаемость мышей, зараженных вирусом псевдобешенства.

Антивирусный эффект обоих веществ автор объясняет их свойством подавлять синтез протеина. По его мнению, клинические результаты, достигнутые с помощью эметина в отношении определенных вирусных заболеваний человека, не могут быть связаны со свойством этого средства подавлять размножение вируса.

PROPERTIES OF L-SERINE DEAMINASE IN SALMONELLA TYPHIMURIUM AND BACILLUS CEREUS

I. RASKÓ, E. KEREKES, L. ALFÖLDI

СВОЙСТВА ЭНЗИМА L-СЕРИНА ДЕАМИНАЗЫ У SALMONELLA TYPHIMURIUM И У BACILLUS CEREUS

И. РАШКО, Е. КЕРЕКЕШ, Л. АЛФЁЛДИ

Энзим L-серин деаминазы у *S. typhimurium* и у *B. cereus* при разведении быстро инактивируется. Инактивирование энзима у *S. typhimurium* разведением может быть замедленно L-серином, а инактивирование энзима у *B. cereus* зависит исключительно от разведения энзима и едва поддается влиянию L-серина. L-серин деаминаза обеих бактерий специфична в отношении L-серина, не разлагает других аминокислот, pH-оптимум 8,5. Потребность в кофакторе выявить не удалось. Их способность разложить субстрат отличается от простой кинетики типа Michaelis-Menten. L-цистеин и D-серин подавляют их активность компетитивным путем.

HEART GLYCOSIDES IN POLIOVIRUS HOST CELL INTERACTION III. CHEMICAL STRUCTURE AND ACTIVITY

A. KOCH, E. GYÖRGY

ВЛИЯНИЕ СЕРДЕЧНЫХ ГЛЮКОЗИДОВ НА ВЗАИМОДЕЙСТВИЕ ВИРУСА ПОЛИОМИЕЛИТА И КЛЕТКИ III. ХИМИЧЕСКАЯ СТРУКТУРА И АКТИВНОСТЬ

А. КОХ, Е. ДЬЁРДЬ

Были изучены следующие химические соединения: дигитоксигенин, дигитоксин, ланатозид А, дигоксин, дигоксигенин, ланатозид С и гитоксин. Каждое из этих соединений понижало число образующихся вирионов и повышало lag-фазу в концентрации 10^{-6} M/l. Самой высокой абсолютной активностью обладали соединения, располагающие структу-

рой типа дигитоксигенин. При концентрации 10^{-7} M/l соединения со структурой дигоксигенина оказались практически неэффективными, тогда как соединения, имеющие структуру дигитоксигенина, подавляли. При 10^{-8} M/l или меньшей концентрации все глюкозиды за исключением гитоксина, а также ланатозид A и C повышали количество вирионов и укорачивали lag-фазу. Из аглюконов дигоксигенин во всех исследованных разведениях понижал число образующихся вирионов, в то время как дигитоксигенин в концентрации 10^{-9} M/l и меньше не оказывал никакого эффекта.

MICROBIOLOGICAL DECOMPOSITION OF 17α-METHYL-17β-HYDROXY STEROIDS WITH ANDROSTANE NUCLEUS

K. G. BÜKI, G. AMBRUS, A. SZABÓ

МИКРОБИОЛОГИЧЕСКОЕ РАЗЛОЖЕНИЕ 17α-МЕТИЛ-17β-ГИДРОКСИ СТЕРОИДОВ С АНДРОСТАНОВЫМ СКЕЛЕТОМ

К. Г. БЮКИ, Г. АМБРУШ, А. САБО

Было проведено сравнение способностей двух хорошо известных микроорганизмов преобразующих стероиды, *Nocardia restrictus* (ATCC 14 800) и ***Mycobacterium phlei***, метаболизировать стероиды со структурой 17α-метил-17β-гидрокси и имеющие андростановый скелет. Оба микроорганизма и эту группу химических соединений разрушают по способу, известному при распаде 17-кетостероидов. В то время как в случае *Nocardia* ***restrictus*** в зависимости от условий можно заменить 9α-гидроксилизацию и Δ1-дегидрогенизацию, ***Mycobacterium phlei*** раньше вносит всегда Δ1-двойную связь. 9α-гидроксилаза у обоих микроорганизмов может быть подавлена 8-гидроксикинолином. В присутствии этого комплексобразующего агента в ферментной жидкости накапливается 17α-метил-17β-гидроксиандроста-1,4 в степени 4-dien-3on из субстратов со структурой 5α-насыщенный-3β-гидрокси, 5α-насыщенный-3-кето, Δ1—5α-насыщенный-3-кето, Δ^4-3-кето, Δ^5-3β-гидрокси и $\Delta^{4,6}$-3-кето.

TRANSFORMATION OF 4,5-EPOXYSTEROIDS WITH MYCOBACTERIUM PHLEI
I. TRANSFORMATION UNDER AEROBIC CONDITIONS

E. TÖMÖRKÉNY, K. ALBRECHT, L. ILA

ТРАНСФОРМАЦИЯ СТЕРОИДОВ 4,5-EPOXI ПОСРЕДСТВОМ MYCOBACTERIUM PHLEI
I. ТРАНСФОРМАЦИЯ В АЭРОБНЫХ УСЛОВИЯХ

Е. ТЁМЁРКЕНЬ, К. АЛБРЕХТ, Л. ИЛА

Стероиды 4,5-epoxi, содержащие 17β-боковую цепочку и имеющие андростановую структуру, в аэробных условиях могут быть трансформированы культурой ***Mycobacterium phlei*** в **androsta-1,4-dien-3,17-dion**. В качестве промежуточного звена удалось выявить **androsta-4-en-3,17-dion**. В процессе трансформации можно было заметить проходящую редукцию 3-кетогруппы и дегидрогенизацию — если присутствует — 17β-гидроксиловой группы.

NEW SELECTIVE MEDIUM FOR THE ISOLATION OF CLOSTRIDIUM PERFRINGENS

M. FÜZI, ZS. CSUKÁS

НОВАЯ СЕЛЕКТИВНАЯ ПИТАТЕЛЬНАЯ СРЕДА ДЛЯ ВЫДЕЛЕНИЯ ШТАММОВ CLOSTRIDIUM PERFRINGENS

М. ФЮЗИ, Ж. ЧУКАШ

Для селективного культивирования ***Clostridium perfringens*** с успехом можно использовать кровяной агар, содержащий 800γ/мл Д-циклосерин битартарата. На селективной питательной среде штаммы ***Clostridium perfringens*** размножаются без помех, Грам-положительные бактерии не растут на ней, и Грам-отрицательные виды также не

образуют колоний, за исключением *Serratia marcescens*. Резистентность Serratia делает возможным и использование питательной среды, проводимое по методу Фортнера, для выделения *Clostridium perfringens*. Селективная питательная среда полностью подавляла смешанную, факультативную аэробную флору фекалий и других выделений различных животных и человека; рост *Clostridium perfringens* наблюдался в чистом виде.

STUDIES ON SWINE SERUM IMMUNOGLOBULINS II. RELATIONSHIP BETWEEN IMMUNOGLOBULINS AND ANTIBODIES

J. SURJÁN

ИССЛЕДОВАНИЕ ИММУНГЛОБУЛИНОВ В СЫВОРОТКЕ СВИНЕЙ II. ВЗАИМООТНОШЕНИЕ МЕЖДУ ИММУНГЛОБУЛИНАМИ И АНТИТЕЛАМИ

Й. ШУРЬЯН

Природа антител β_2M и β_2A глобулиновых фракций, считаемых иммунглобулинами, была доказана на основе их непосредственной реакции, вызванной их антигенами. Так, преципитины сыворотки свиней, хорошо преципитирующие некоторые антигены бактериального происхождения, оказались иммунглобулинами IgM и IgA.

Макроглобулин IgM и в процессе образования иммунитета у свиней можно было раньше выявить, чем иммунглобулин IgA. Опыты подтвердили, что в определенную стадию иммунитета в организме против одного антигена могут и одновременно присутствовать два вида антител, специфичность которых одинаковая, однако, их иммунглобулиновые свойства различны.

STUDIES ON PERITONEAL EXUDATE OF ANIMALS EXPERIMENTALLY INFECTED WITH TOXOPLASMA GONDII II. SUSCEPTIBILITY OF RATS AND CHICKENS

M. A. SAMIR

ИССЛЕДОВАНИЕ ПЕРИТОНЕАЛЬНОГО ЭКССУДАТА ЖИВОТНЫХ, ЭКСПЕРИМЕНТАЛЬНО ЗАРАЖЕННЫХ TOXOPLASMA GONDII II. ЧУВСТВИТЕЛЬНОСТЬ КРЫС И ЦЫПЛЯТ

М. А. САМИР

Цыплятам внутривенно вводили, постепенно увеличивая дозу, перитонеальный экссудат мышей, зараженных токсоплазмой. Соответствующие дозы экссудата вызывали нарушения со стороны дыхания и нервной системы и даже гибель животных. Крысы также оказались чувствительными при внутривенном введении. При внутрибрюшном введении экссудат не вызывал никаких симптомов ни у цыплят, ни у крыс.

Замораживание и оттаивание понижали токсичность экссудата для крыс, но не снимали её.

COLICIN SENSITIVITY AND COLICINOGENITY OF TYPE O124:K72(B17) E. COLI STRAINS

K. CSISZÁR

ЧУВСТВИТЕЛЬНОСТЬ К КОЛИЦИНУ И КОЛИЦИНОГЕННОСТЬ ШТАММОВ E. COLI СЕРОТИПА O124 : K72 (B17)

К. ЧИСАР

С точки зрения чувствительности к колицину и колициногенности было изучено 505 штаммов *E. coli* O124, происходящих из различных областей Венгрии. 54,2% штаммов оказались колициногенными. В 96% штаммы были чувствительны к 2 (или больше) колицинам. На основе исследований удалось отличить 31 тип. Автор считает колициновую типизацию полезным методом для эпидемиологического анализа штаммов *E. coli* O124.

ANTIGENIC RELATIONSHIP BETWEEN SHIGELLA SONNEI PHASE II AND ENTEROBACTER CLOACAE

B. RALOVICH, S. VÖRÖS, A. VERTÉNYI

АНТИГЕННАЯ СВЯЗЬ МЕЖДУ II ФАЗОЙ SH. SONNEI И ENTEROBACTER CLOACAE

Б. РАЛОВИЧ, Ш. ВЁРЁШ, А. ВЕРТЕНЬИ

Авторы выделили штамм *Enterobacter cloacae*, обозначенный как 6302/RB, который имеет жгутиковый, поверхностный (L) и соматический (O) антигены. Было установлено, что за счёт О-антигена штамма антиген характера «ав-ас» находится в родственной связи со II фазой *Sh. sonnei*. Обсуждаются диагностические проблемы, связанные с полученными результатами.

PHAGE AND COLICIN TYPING OF SHIGELLA SONNEI

V. G. LÁSZLÓ, L. KEREKES

ФАГ- И КОЛИЦИН-ИССЛЕДОВАНИЯ У ШТАММОВ SHIGELLA SONNEI

В. Г. ЛАСЛО, Л. КЕРЕКЕШ

Авторы определили тип фага у 3522 штаммов и на основе колициногенных свойств тип колицина у 1418 штаммов *Shigella sonnei*, выделенных из различных районов Венгрии. Типы, на основе повторных определений **in vivo** и **in vitro**, оказались постоянными. Типы по фагу и колицину в течение теста шегеллёзного кератоконъюнктивита не изменялись.

Наиболее частыми фаговыми типами были 6-ой, 7-ой и 65-ый, наиболее частыми типами по колицину — 0-ой, 6-ой и 4-ый.

Некоторые фаговые типы типизацией по колицину могли быть подразделены на последующие группы, и, таким образом, авторы нашли приемлемым для эпидемиологических целей совместное применение обоих методов.

INTERFERON STIMULATION AND OTHER PHENOTYPIC PROPERTIES OF TWO VARIANTS OF POLYOMA VIRUS

M. TÁLAS

ИНТЕРФЕРОНСТИМУЛИРУЮЩАЯ СПОСОБНОСТЬ И ДРУГИЕ ФЕНОТИПИЧЕСКИЕ СВОЙСТВА ДВУХ ВАРИАНТОВ ВИРУСА ПОЛИОМЫ

М. ТАЛАШ

Автором изучались два штамма вируса полиомы (**Toronto-26** и **SE-55**) и было установлено, что они отличаются друг от друга не только по их онкогенной активности на новорожденных хомячках, но и по другим свойствам, которые можно было наблюдать *in vitro* в клеточной культуре эмбриона мыши и хомяка.

Вирусный штамм Т—26, обладающий более выраженной онкогенностью, стимулировал в клеточной культуре эмбриона мыши и хомяка более быстрое образование интерферона и с более высоким титром, чем штамм SE-55, обладающий низкой онкогенной активностью.

В клеточной культуре эмбриона мыши для штамма Т—26 оказался характерным более медленный синтез инфекционного вируса и более медленное развитие цитопатического эффекта, а также более высокая гемагглютинирующая активность в противоположность таковым штамма SE-55.

Обсуждается значение связи между онкогенной активностью и способностью двух вариантов вируса полиомы индуцировать интерферон.

SEROLOGICAL AND PHAGE TYPING OF PSEUDOMONAS AERUGINOSA INVADING A MUNICIPAL WATER SUPPLY

J. LANTOS, M. KISS, B. LÁNYI, J. VÖLGYESI

ИДЕНТИФИКАЦИЯ СЕРО- И ФАГ-ТИПИЗАЦИЕЙ ШТАММОВ PSEUDOMONAS AERUGINOSA, ВЫРАЩЕННЫХ ИЗ ВОДНОЙ СЕТИ

Й. ЛАНТОШ, М. КИШШ, Б. ЛАНЬИ, Й. ВЁЛДЬЕШИ

Серо- и фаг-типизацией выращенных штаммов было раскрыто происхождение массивного загрязнения в 1967—68 гг. ***Ps. aeruginosa*** водной сети в городе Сегед.

В связи с инвазией водной сети были проведены попытки выделения агента; в 34,5% водных проб сети и 11,7% водных проб, взятых на территории подозреваемой водной станции, удалось выделить штаммы, относящиеся к одной и той же серогруппе (4а, 4с), фаговая чувствительность которых также была одинаковой. С удалением источника загрязнения прекратилось и загрязнение водной сети с ***Ps. aeruginosa***.

PROPERTIES OF ACETOHYDROXYACID SYNTHETASE IN MYCOBACTERIUM PELLEGRINO

A. SZENTIRMAI, I. HORVÁTH

СВОЙСТВА СИНТЕТАЗЫ АЦЕТОГИДРОКСИ-КИСЛОТЫ, ЭКСТРАГИРОВАННОЙ ИЗ MYCOBACTERIUM PELLEGRINO

А. СЕНТИРМАИ, И. ХОРВАТ

Свойства синтетазы ацетогидрокси-кислоты, экстрагированной из *Mycobacterium pellegrino*, её потребность в коэнзиме подобны свойствам биосинтетического энзима, экстрагированного из других бактерий; подавляется валином.

Незначительные воздействия, разведение, pH выше 8,0, высокая (выше 15 мМ) концентрация субстрата, температура выше 33° в присутствии субстрата медленно инактивируют энзим. Перед инактивацией наблюдается проходящий рост активности, который сопровождается понижением чувствительности к валину. Против всех перечисленных воздействий валин оказывает защитный эффект.

Кривая насыщения субстрата имеет характер сигмоида, но в области 2—15 мМ концентрации субстрата имеет вид гиперболы, а значение K_m периода, высчитанное на основе этой фазы, равно 1,61 мМ. В присутствии валина сигмоидный характер исчезает. Кроме валина, и кофакторы стабилизируют энзим.

INDUCTION AND MULTIPLICATION OF λ-PHAGE IV. THE EFFECT OF PANFURAN

I. HORVÁTH, I. OTT, I. GADÓ

ИНДУКЦИЯ И РАЗМНОЖЕНИЕ λ-ФАГА IV. ДЕЙСТВИЕ ПАНФУРАНА

И. ХОРВАТ, И. ОТТ, И. ГАДО

Панфуран, 3-амино-6(2)5 нитро-2-фурил(винил)1,2,4 триазин HCl, подавляющий синтез ДНК, в концентрации 0,03 μг/мл индуцирует штамм *E. coli* К12(λ—28) с кинетикой, подобной таковой у митомицина. Варианты, резистентные к мицину, панфурану и гидроксиуреи, с точки зрения фаговой индукции не показывали перекрестной резистенции.

AMINO ACID UTILIZATION BY SEROLOGICALLY GROUPED PSEUDOMONAS AERUGINOSA STRAINS

B. LÁNYI

УТИЛИЗАЦИЯ АМИНОКИСЛОТЫ СЕРОЛОГИЧЕСКИ СГРУППИРОВАННЫМИ ШТАММАМИ PSEUDOMONAS AERUGINOSA

Б. ЛАНЬИ

В определенной питательной среде была изучена утилизация аминокислоты 233 штаммами *Ps. aeruginosa*, относящимися к 23 различным серологическим группам, выбранными по частоте серологических групп и происходящими из различных источников.

Спектр аминокислоты *Ps. aeruginosa* может быть охарактеризован следующим образом: Л-аланин 99,6%, гамма-аминомасляная кислота 99,6%, Л-аргинин 99,6%, ДЛ-аспарагиновая кислота 99,6%, ДЛ-глютаминовая кислота 99,6%, глицин 99,6%, L-гистидин 99,6%, гидрокси-Л-пролин 99,6%, ДЛ-изолейцин 98,3%, ДЛ-лейцин 98,3%, Л-лизин 99,6%, ДЛ-орнитин 99,6%, ДЛ-фенилаланин 99,6%, Л-пролин 99,6%, Л-триптофан 97,4%, Л-тирозин 99,6%, ДЛ-валин 99,6 — положительно. Л-цистеин, Л-цистин и ДЛ-метионин 100% — отрицательно. Неопределенные и даже для одного и того же штамма меняющиеся результаты были получены на питательной среде, содержащей ДЛ-норлейцин, ДЛ-норвалин, ДЛ-серин и Л-треонин.

По полученным результатам, *Ps. aeruginosa* согласно спектру аминокислоты представляет собой гомогенный вид.

THE EFFECT OF HYPOTHERMIA ON ALLERGIC-TYPE SKIN REACTIONS

T. SZILÁGYI, H. CSERNYÁNSZKY, S. IMRE, M. WENT

ВОЗДЕЙСТВИЕ ГИПОТЕРМИИ НА КОЖНУЮ РЕАКЦИЮ АЛЛЕРГИЧЕСКОГО ХАРАКТЕРА

Т. СИЛАДЬИ, Х. ЧЕРНЬАНСКИ, Ш. ИМРЕ, М. ВЕНТ

Авторы вызывали на мышах пассивную кожную анафилаксию и динитрохлорбензоловую гиперчувствительность, а на морских свинках — инвертный пассивный феномен Arthus-а. Было установлено, что при вызывании реакции у животных, находящихся в охлажденном состоянии, симптомы или вообще не появляются, или они выражены значительно слабее, чем у контрольных животных с нормальной температурой. В статье обсуждается патомеханизм кожных реакций и подавляющий эффект гипотермии.

COMPARATIVE STUDIES ON THE TAXONOMIC RELATIONSHIP BETWEEN MYCOBACTERIUM ABSCESSUS AND MYCOBACTERIUM BORSTELENSE. INCIDENCE OF MYCOBACTERIUM ABSCESSUS IN MONKEYS

J. G. WEISZFEILER, V. KARASSEVA, E. KARCZAG

СРАВНИТЕЛЬНОЕ ИССЛЕДОВАНИЕ MYCOBACTERIUM ABSCESSUS и MYCOBACTERIUM BORSTELENSE И ИХ ТАКСОНОМИЧЕСКАЯ ВЗАИМОСВЯЗЬ. ОБНАРУЖЕНИЕ M. ABSCESSUS В ОБЕЗЬЯНАХ

И. Г. ВЕИСФЕЙЛЕР, В. КАРАСЁВА, Е. КАРЦАГ

1. *Mycobacterium abscessus* по своим биохимическим и патогенным свойствам, а также по антигенной структуре представляет собой хорошо определенный вид, не имеет серологического родства с *Mycobacterium fortuitum* и не может рассматриваться как подвид последней. На основании серологической группировки микобактерий относятся в группу Д.

2. *Mycobacterium abscessus* и *Mycobacterium borstelense* с точки зрения их биохимических свойств и антигенной структуры являются идентичными, но отличаются по их патогенности для мышей, поэтому они рассматриваются авторами как различные виды, но относящиеся в одну серологическую группу.

3. Штаммы, обозначенные как *Mycobacterium runyonii*, по всем свойствам оказались идентичными с *M. abscessus*.

4. Два штамма, М4 и М15, выделенные авторами от обезьян *Macaccus rhesus*, по биохимическим, патогенным свойствам и антигенной структуре оказались идентичными с *M. abscessus*. Штамм М15, введенный в морской свинке, не даёт защитного эффекта против турбекулёза, даже сенсибилизирует животное в отношении вирулентного заражения.

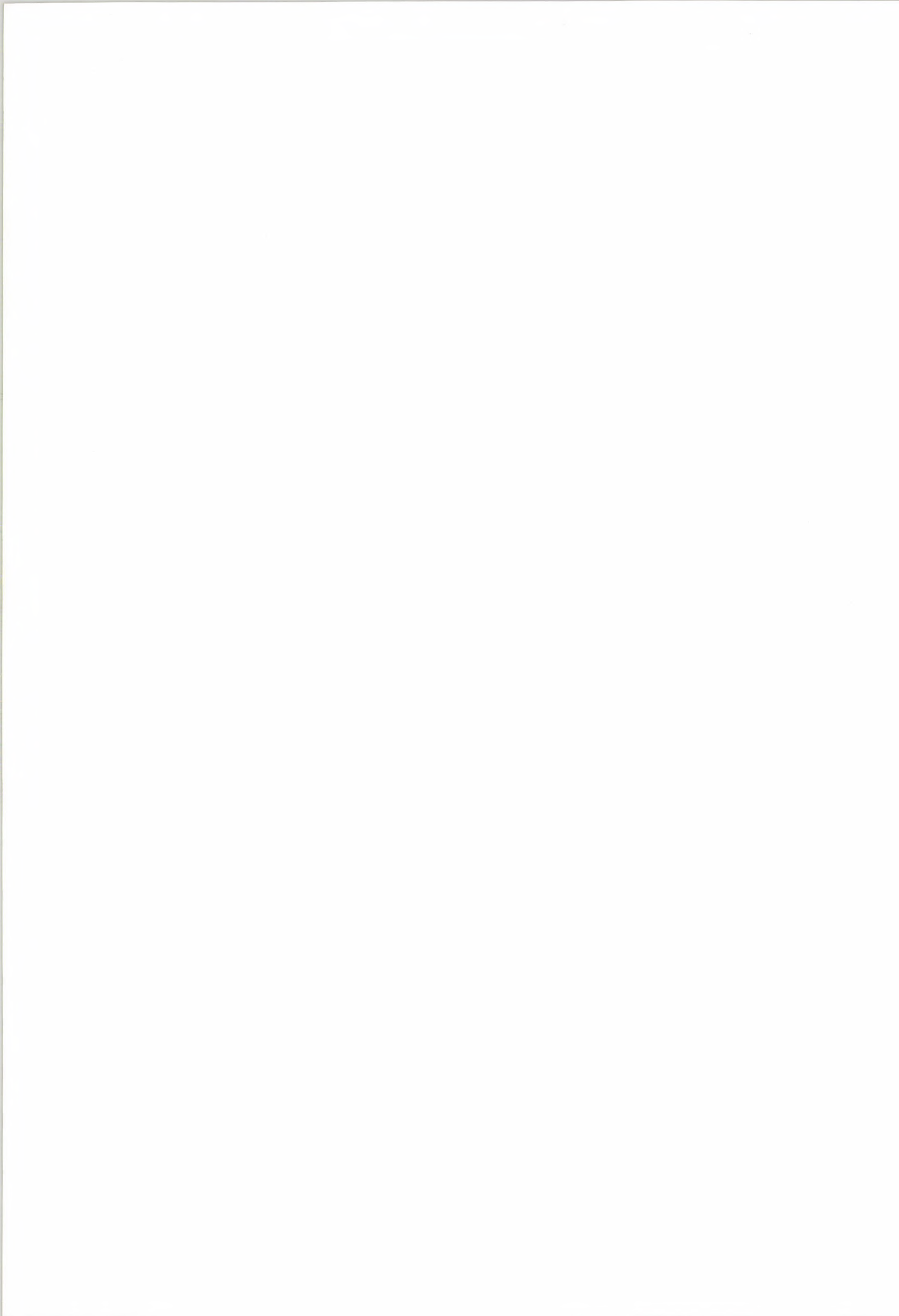
INTESTINAL MICROFLORA OF THE LARVAE OF ST. MARK'S FLY IV. STUDIES ON THE INTESTINAL BACTERIAL FLORA OF A LARVA POPULATION

I. SZABÓ, MARIA MARTON and ILONA

ИНТЕСТИНАЛЬНАЯ МИКРОФЛОРА ЛАРВ МАРТОВСКОЙ БАРХАТНОЙ МУХИ. IV. ОПЫТЫ ПО БАКТЕРИАЛЬНОЙ ФЛОРЕ ПОПУЛЯЦИИ ЛАРВ

И. САБО, М. МАРТОН, И. БУТИ

В кишечном тракте ларв мартовской бархатной мухи происходит сильная селекция бактериальной флоры, накопленной почвой и органическими остатками, поступившими с пищей. Абсолютно доминирующий вид бактериальной популяции, представляющий более чем 70% интестинальной микрофлоры, был *Pseudomonas fluorescens*. Наряду с этим значительную роль играет *Enterobacter aerogenes*, встречающийся в почве только в следах, далее, неопределенный промежуточный тип из семьи Enterobacteriaceae. Некоторые представители почвенной микрофлоры, устойчивые к пассажу в кишечнике (*Bacillus cereus*, *Mycobacterium* sp., *Chromobacterium* sp. и др.), проходят по пищеварительному тракту с большей или меньшей редукцией их числа. Другие, в первую очередь господствующие представители уровней A_H и A_F (*Bacillus subtilis*, *Bacillus sphaericus*, *Alcaligenes faecalis*, *Achromobacter pestifer*, *Agrobacterium radiobacter*, *Rhizobium* spp., *Micrococcus cryophilus* и др.), уже не могут быть обнаружены в средних и конечных отделах кишечника, а также в свежих экскрементах. Хотя картина вышеизложенной селекции характерна, но не может считаться общей, так как факторы окружающей среды (пища ларв, тип почвы и т. д.) в значительной степени влияют на качественный и количественный состав доминирующей бактериальной флоры кишечника, что ведет за собой и изменения в возможности переживания проходящей микрофлоры почвы.



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