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GENETIC RELATIONSHIPS BETWEEN MYCOBACTERIUM KANSASII AND MYCOBACTERIUM TUBERCULOSIS*

By

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(Received July 3, 1963)

Summary. Examination of the biochemical, pathogenic, serological and immunogenic properties of two *M. kansasii* strains showed that their properties were similar to those of *M tuberculosis*, with resistance against isoniazid and attenuated virulence. R variants werd obtained as a result of dissociation, from both strains. The R variant of the POLLAK ane BUHLER's strain proved to be an attenuated strain of *M. tuberculosis*, both in cultural and immunogenic properties. According to the authors' opinion this finding allows the conclusion that *M. kansasii* has arisen by mutation from *M. tuberculosis* and that, by virtue of its human pathogenicity, it constitutes a new type of tubercle bacillus.

In the last ten years, studies on a typical mycobacteria isolated from human sources resulted in the differentiation of a new Mycobacterium species by POLLAK and BUHLER [1]. The culture produced no pigment in the dark, but after a short exposure to light its colour turned into yellow; in the guinea pig, the organism caused no progressive disease. Later it was shown that the yellow bacillus differed from the typical human tubercle bacillus in the lack of niacin formation. Subsequently, several strains with similar properties were cultured [2—5, 22]. TIMPE and RUNYON [6] classified these mycobacteria as photochromogenic strains. The organism was recognized as *M. kansasii* by the Subcommittee on Mycobacteria, American Association for Microbiology [7]. The origin of *M. kansasii*, however, is not clear. The question arises whether the organism has been existing for a long time, causing disease for centuries and thousands of years, as the causative agents of tuberculosis and leprosy did, or, else, *M. kansasii* developed as a result of the variation of *M. tuberculosis* in each case and is unable to spread among human beings through several generations. Would the latter consideration be true, it would explain why these organisms occur more frequently as a result of chemotherapeutic treatment. Due to a primary resistance to streptomycin, PAS and isoniazid, the disease caused by the microorganism responds less to treatment and thus the attention of physicians is called to such particular cases. In the debate concerning the problem, RUNYON [8] claimed that *M. kansasii* was a species independent from *M. tuberculosis*. In contrast, according to THARSIS [9] and XALABARDER [10] these strains are closely related to *M. tuberculosis*.

* Partly presented at the 9th International Conference against Tuberculosis, Rome, 1963

In studying the variation of *M. tuberculosis*, we found [11] that strains with different hereditary properties might arise both *in vitro* and *in vivo*, and their relationship to *M. tuberculosis* could be detected immunologically. Examination of the immunogenicity of such strains against tuberculosis seemed especially significant. The usefulness of serological methods in demonstrating biological relationship is well known and the detection of common antigens by gel precipitation is considered a particularly important method [12]. The determination of the biochemical properties for the identification of various strains is also of basic importance in the classification of mycobacteria [13].

Materials and methods

Two *M. kansasii* strains were examined. (i) Strain 232, the yellow bacillus of POLLAK and BUHLER, regarded as the standard strain, was received from Prof. P. HAUDUROY. (ii) Strain 5792 was received from Prof. G. MEISSNER. Strain 232—R derived from strain 232 by dissociation will also be dealt with in this paper. Growth characteristics at 37° C and room temperature, pigmentation and colony morphology were observed. Enzyme activity against different amides, the presence of nitrate reductase, aryl sulphatase, catalase and peroxidase, and the niacin reaction were examined. Susceptibility testing was carried out against streptomycin, isoniazid, PAS and thiosemicarbazone on Löwenstein's medium at concentrations of 1, 10 and 50 μg/ml. Pathogenicity was tested in rabbits (20 mg, intravenously), guinea pigs (1 mg, subcutaneously) and mice (1 mg, intravenously), partly by recording the number of deaths, partly by sacrificing the animals 30—60 days after inoculation. In some cases cultures were made from the organs and pus. Immunogenicity of the strains was examined in guinea pigs, with 1 mg doses. Strain H 37 Rv 0,00001 mg was inoculated for superinfection. A detailed description of this method has been published previously [14]. Comparison of the antigenic structure was performed by the gel-precipitation method, combined with immune-electrophoresis.

Results

After 2 years of subculture on glycerol-potato, both strains (232 and 5792) produced white rough colonies in the smooth, pigment-producing culture, allowing to isolate photochromogen rough variants. In 4 cultures obtained from the lymph glands of guinea pigs inoculated with strain 232, after

Table I

Biochemical properties of M. kansasii strains and M. tuberculosis strain H 37 Rv

	M. tubercul.	*M. kansasii*		
	H 37 Rv	232	232 R	5792
Urease	+	+	+	+
Nicotine amidase ..	+	+	+	+
Nitrate reductase ..	+	+	+	+
Catalase	+	+	+	+
Peroxidase	+	+	+	+
Arylsulphatase ...	+	+	+	+
Niacin	+	—	—	—

Table II

Immunological properties of M. kansasii strains and the attenuated M. tuberculosis strains BCG and 115

Experiments	Strain	No. of animals	Tuberculous index
I	*M. kansasii* N 232	9	6.4
	M. tuberculosis N 115	10	8.0
	Control	10	19.9
II	*M. kansasii* N 232 R	10	6.3
	M. kansasii 5792	10	6.6
	M. tuberculosis BCG	9	7.8
	Control	10	19.3

exposure to light, some white R colonies were observed among the pigmented S colonies. The isolated 232—R strain was examined for biochemical, pathogenic and immunogenic properties.

As shown by Table I, in enzyme activity the two authentic *M. kansasii* strains and the 232 R variant were similar to *M. tuberculosis*. The only difference from the human tubercle strain was that our strains produced no niacin.

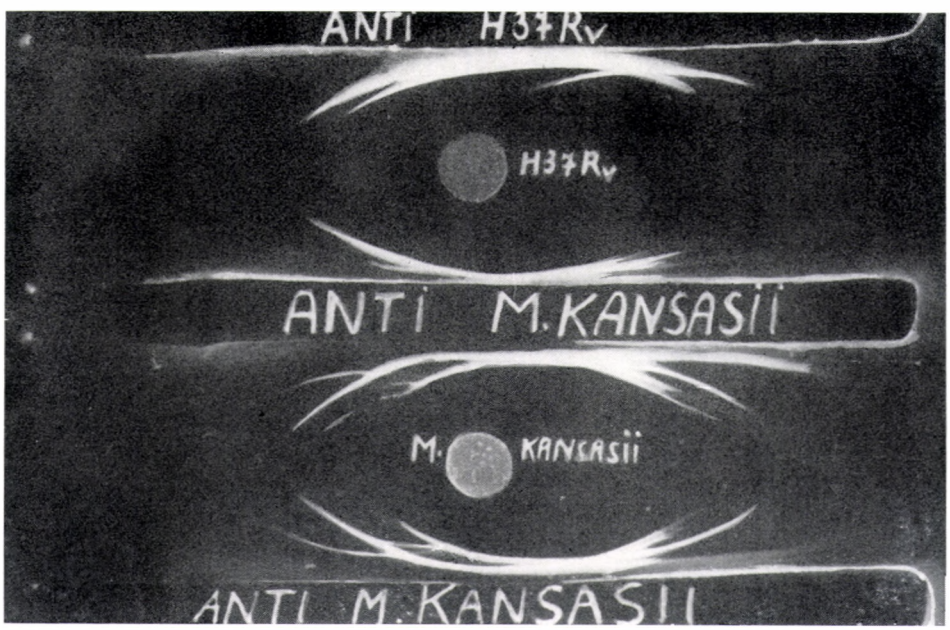

Fig. 1. Immunoelectrophoretic precipitation lines in homologous and heterologous systems Antigens: *M. tuberculosis* H 37 Rv, *M. kansasii* 232. Antisera: Anti H 37 Rv and Anti *M. kansasii*

In Table III the sensibility against isoniazid, streptomycin, PAS and thiosemicarbazone of the cultures is presented. All the three strains were resistant to 10 μg/ml of streptomycin and PAS, moderately resistant to the same concentration of isoniazid, and sensitive to 10 μg/ml of thiosemicarbazone. The cultures in doses of 20 mg caused fatal disease in rabbits involving severe pulmonary lesions similar to those induced by the BCG and 115 strains [15]. In guinea pigs at the site of the injection abscesses and in the regional lymph glands caseous changes, in white mice within 30—40 days progressive changes in the lungs and kidneys occurred.

Table III

Growth on media containing various concentrations of chemotherapeutic substances

Strain	Streptomycin			INH			PAS			Thiosemi-carbazone		
	1	10	50	1	10	50	1	10	50	1	10	50
	μg/ml			μg/ml			μg/ml			μg/ml		
H 37 Rv	−	−	−	−	−	−	−	−	−	±	−	−
M. kansasii 232	+	+	−	+	±	−	+	+	+	±	−	−
M. kansasii 232 R	+	+	−	±	±	−	+	+	+	+	−	−
M. kansasii 5792	+	+	−	±	±	−	+	+	−	+	−	−

The three strains were found to be equivalent in immunogenic value (Table II) to the BCG strain and WEISSFEILER's attenuated tubercle bacillus strain No. 115[14].

The study of the antigenic structure revealed that *M. kansasii* has 6 antigens in common with *M. tuberculosis*.

Discussion

The biochemical, pathogenic and immunological characteristics of the investigated *M. kansasii* strains are strikingly similar to the corresponding properties of *M. tuberculosis* of attenuated virulence. INH resistant tubercle bacillus strains often show an attenuated virulence for guinea pigs; in mice, however, they retain their virulence. The close relationship is indicated by the antigenic structure, too, *M. kansasii* having 6 antigens in common with *M. tuberculosis* and only 2—4 with saprophytic Mycobacteria.

The formation by dissociation of an R variant is particularly important. The phenomenon should be regarded as a mutation resulting in the production of a strain possessing the cultural and immunological properties of *M. tuberculosis*. It might, however, be argued that the R variant has been present in a latent form in the original strain 232, in view of the fact stressed by HAUDUROY [16] that certain strains contain a heterogenic population, from

which the latent forms can be cultivated. However, neither of our two strains gave off R colonies when examined by a simple spreading technique.

When comparing our experiments with the data in the literature, it should be noted that FREERKSEN [17], KLUGH and PRATT [18], in guine pigs, YOUMANS, PARLETT and YOUMANS [19] in white mice showed the photochromogenic strains to have a strong immunogenic potency against tuberculosis. PARLETT and YOUMANS [20] established the presence of common antigens in the photochromogenic strains and *M. tuberculosis*. MITCHISON and SELKON[21], WAYNE [5] observed *M. kansasii* in patients previously excreting *M. tuberculosis* in the sputum. Clinically, the disease caused by *M. kansasii* cannot be differentiated from tuberculosis, and the histological changes are similar in both diseases.

Our observations have thus furnished proof of a close relation between *M. kansasii* and *M. tuberculosis*. The R variant of strain 232 shows the cultural, biochemical and immunogenic characteristics of attenuated *M. tuberculosis* strain, with resistance against the usual chemotherapeutic substances. It is known that the bovine type of tubercle bacillus do not produce niacin. Accordingly we suppose that *M. kansasii* has developed as a result of the variation of *M. tuberculosis*, retaining most of the biochemical, serological, pathogenic and immunogenic properties of the parent organism. The fact that *M. kansasii* is pathogenic and actually causes disease in man, and perhaps is adapted to spreading among humans, justifies its separation as a new type of tubercle bacillus. As the American Classification recognises the bovine and murine types of tubercle bacillus as independent species, *M. kansasii* might also be regarded as a new species.

Modern chemotherapy, influencing not only the survival but also the variation of *M. tuberculosis*, may increase the incidence of *M. kansasii* and the severity of the caused disease. As the organism is resistant to the major chemotherapeutics, the disease is difficult to treat. This means that the organism will probably occur with higher frequency in the future. Therefore, the knowledge of the agent's origin and the establishment of preventive measures against the disease caused by it, are considered of great importance.

Acknowledgement is made to Mrs. K. BIHARI for technical assistance.

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SEROLOGICAL ACTIVITY OF MYCOBACTERIAL DESOXYRIBONUCLEIC ACID PREPARATIONS

I. EFFECT OF PURIFICATION AND HEATING ON THE COMPLEMENT FIXATION BY DESOXYRIBONUCLEIC ACID

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(Received July 9, 1963)

Summary. Complement fixation caused by protein, polysaccharide and perhaps ribonucleic acid contamination in different mycobacterial desoxyribonucleic acid preparations has been found to be eliminated by phenol-chloroform-amyl alcohol purification. Complement fixation by preparations purified in this manner or chromatographed on methylated albumin can be decreased by desoxyribonuclease but not by carboxypeptidase, ribonuclease, trypsin or lysozyme.

Complement fixation by heated and rapidly chilled desoxyribonucleic acid solutions shows a characteristic temperature dependence. Serological activity exhibits maximum and minimum values at several temperatures. The variation in activity may be explained by a "surface" alteration of the antigen. No direct correlation has been shown between the increased U. V. absorption at 254 mμ and serological activity of the heated preparations.

The possible presence in desoxyribonucleic acid preparations of contaminating materials not detectable by chemical or enzymic decomposition, has been suggested.

In a previous paper the variation of serological activity on heating of enzymically digested mycobacterial desoxyribonucleic acids (DNA) has been discussed [1]. The serological activity of highly purified mycobacterial DNA preparations has also been investigated [2, 3].

In this regard it was of interest to establish that step of the isolation and purification procedure, in which the role of DNA in complement fixation can be undoubtedly confirmed. In these experiments a decreased complement fixation after desoxyribonuclease (DNase) treatment was regarded as a criterion of serological effectiveness.

In addition, it seemed desirable to investigate the serological activity of heated preparations obtained with different purification procedures.

Materials and methods

Bacteria. Cultures and cultivation methods have been described previously [2].

Antigens. Bacterial homogenates were prepared as detailed previously [2, 3]. DNA preparations were produced from *M. friburgensis* and *smegmatis* by the combined method of KIRBY [4] and SEVAG *et al.* [5], as follows.

The bacteria were washed in distilled water, then 100 g amounts (wet weight) were ground with glass powder in a mortar. After adding 100 ml 6 per cent sodium p-aminosalicylate in several fractions, the homogenate was mixed with 100 ml 90 per cent phenol and then shaken for one hour. After centrifugation for 20 minutes at approximately 8000 r. p. m., 1 part of the aqueous phase was mixed with 1 part of ethanol (v/v) and the precipitate was immediately dissolved in 0.15 *M* NaCl. The precipitate will be referred to as "crude DNA preparation".

The crude preparation was purified in three different ways.

DNA preparation A : The crude preparation was dissolved in 0.15 *M* NaCl and, after adding 1 volume 3 : 1 chloroform-amyl alcohol mixture, it was shaken for 20 minutes. After centrifugation shaking with the above mixture was repeated. Following a subsequent centrifugation, DNA was precipitated from the aqueous phase with one volume ethanol, then the precipitate was washed twice in ethanol. Prior to use the preparation was stored at 0° C under 95 per cent ethanol.

DNA preparation B : The crude DNA was dissolved in 0.15 *M* NaCl, then precipitated with ethanol. The precipitate was redissolved and the solution shaken for 15 minutes with one

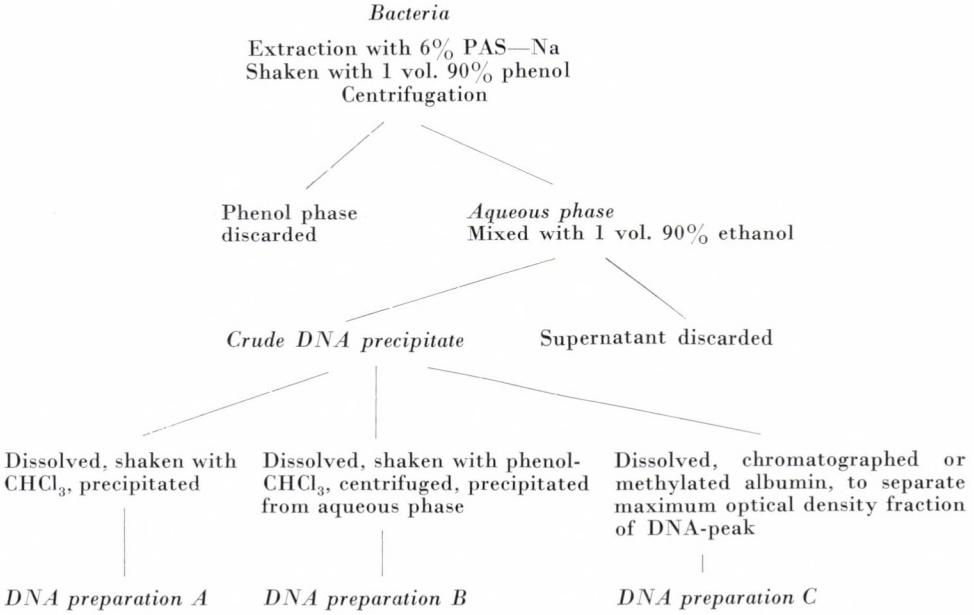

Fig. 1. Preparation scheme for *M. friburgensis* and *M. smegmatis DNA*

volume of a mixture of 90 per cent phenol : chloroform : amyl alcohol (7 : 2 : 1). After centrifugation at 8000 r. p. m., DNA was precipitated with one volume ethanol. Washing in ethanol was continued until all the phenol had been removed. Prior to use the preparation was stored at 0° C under 95 per cent ethanol.

DNA preparation C : The crude DNA dissolved in 0.15 *M* NaCl was chromatographed as described by SUEOKA and CHENG [9]. For the serological reactions, the fraction of "DNA-peak" giving maximum optical density values was used without the isolation of DNA. The steps of purification are presented in Fig. 1.

Enzymic treatment of DNA preparations. Commercial DNase, trypsin, lysozyme, ribonuclease (RNase) and carboxypeptidase were used. The activity of the enzymes was tested in preliminary examinations.

Trypsin, lysozyme and RNase were dissolved in 0.15 *M* NaCl, carboxypeptidase in 0.15 *M* NaCl containing 0.15 *M* Na_2HPO_4. DNase was dissolved in a solution containing 0.008 *M* $MgSO_4$, 0.15 *M* NaCl and 0.012 *M* sodium acetate.

All enzymes were used at concentrations of 50 μg/ml.

To 5 ml 0.15 *M* NaCl solution of DNA, 5 ml enzyme was pipetted. The mixture was incubated for 2 hours at 37° C and subsequently for 16 hours at 0° C, then the complement fixation test was carried out. Decomposition of DNA was estimated by measuring the increase in optical density at 254 mμ. Heating experiments were carried out in sealed 2 ml ampoules, each containing 0.10 ml of the DNA—enzyme system. Controls for the serological test contained only the DNA preparation and solvents without the enzymes.

Heating experiments. The sealed solutions were kept at 0° C prior to heating. In case of enzymic digestion experiments the ampoules contained also the corresponding enzyme. The

ampoules were immersed in a water bath of the required temperature for 15 minutes. After the heating period the ampoules were immediately cooled to 0° C and stored at that temperature until used. This method is a modification of STOLLAR and LEVINE's technique [12].

Heating and serological experiments were performed in the following order.

First, the preparation was digested and distributed in ampoules. Next day the ampoules were exposed to heat. On the third day the complement fixation tests were carried out. The same results were obtained when these procedures were completed within one day. The three-day period was chosen only for practical purposes.

When heating was not required, the DNA solutions were not distributed into ampoules. In such experiments dilution of the antigen was made directly from the stock solution.

Serological reaction. Complement fixation was performed as described previously [3], with the modification that, instead of veronal buffer, 0.15 *M* NaCl was used. The optimal antigen concentration for testing heated preparations was determined in antigen and serum dilution series. Every experiment was checked by suitable controls.

Analytical examinations. Nitrogen and phosphorus were determined by the technique of MARKHAM [6] and of MARTLAND and ROBISON [7]. The method of CHARGAFF and ZAMENHOF [8] served for estimating the $\varepsilon_{(P)}$ value. Chromatography was carried out by the methods of SUEOKA and CHENG [9] or of MANDELL and HERSHEY [27]. The T_m value was estimated as described by MARMUR and DOTY [13, 14]; in lack of a suitable adapter, instead of heating the material in the cuvettes, aliquots of continuously heated DNA solutions were measured at the required intervals.

The analytical data are summarized in Table I.

Table I

Analysis of DNA preparations

DNA preparation	Method of purification	N/P	$\varepsilon_{(P)}$	Approximate RNA content %	Approximate protein content %	Glucose content of hydrolysate %
"Crude"	—	2.4	3200	15	5	5
A	$CHCl_3$	1.72	6200	15	1	< 1
B	Phenol—$CHCl_3$	1.75	7450	15	1	< 1
C	Chromatography	.	6900	.	.	.

. = Not tested

Rabbits were immunized with bacterial homogenates as described previously [1, 2]. The sera were inactivated at 56° C for 30 minutes then stored at −20° C.

Experimental data. Instead of calculating the amount of complement from the percentage of haemolysis according to the VAN KROGH equation [11], the result was expressed in 100 − haemolysis % units. Complement fixation by heated DNA preparations was expressed as

$$\text{Relative activity} = \frac{\text{Intensity of photocurrent for DNA kept at } 0^\circ \text{ C}}{\text{Intensity of photocurrent for DNA heated to } t^\circ \text{ C}}$$

The intensity of photocurrent is a function of the haemoglobin concentration in th supernatant of the complement fixation system, and thus an indicator of the degree of haemolysis.

Results

Complement fixation by crude DNS prepared from *M. friburgensis* was decreased by any of the examined enzymes. The most definite decrease was observed with carboxypeptidase (Fig. 2). Chemical analysis revealed that the

preparation contained protein, polysaccharide and RNA. The decreasing effect of RNase indicates the possible serological activity of RNA. Studies on this problem are in progress.

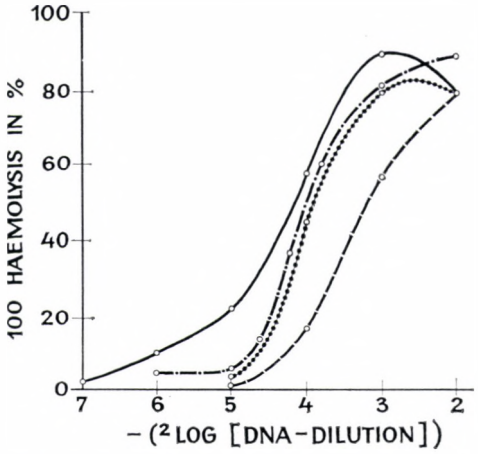

Fig. 2. Complement fixation by crude DNA preparation and 1 : 100 diluted anti-friburgensis serum

o—o DNA; o- - -o DNA digested with carboxypeptidase;
o·····o DNA digested with RNase; o· — · —o DNA digested with DNase

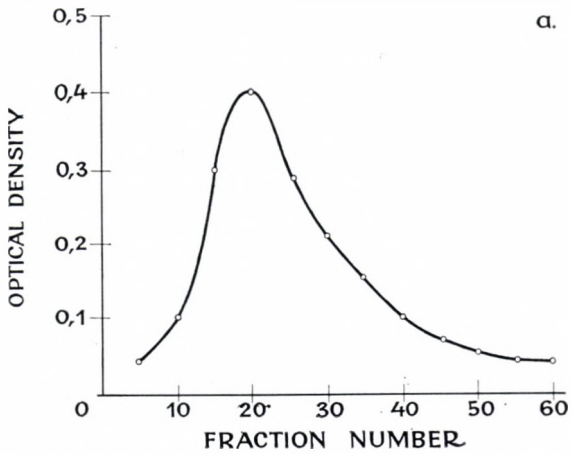

Fig. 3a. Chromatography of preparation A

Chloroform-treated *M. friburgensis* preparation A contained protein and RNA. As concluded from the enzymic digestion, the carboxypeptidase sensitive component played an active part in complement fixation. The activity of the DNase sensitive fraction was more definite than that of the crude preparation.

When preparation A was heated to 56° C or over, the relative activity of the antigen changed as shown in Fig. 3c. After DNase digestion and heating, the serological activity decreased. Decomposed DNA also yielded a peak at 93° C, similarly to the undigested preparation.

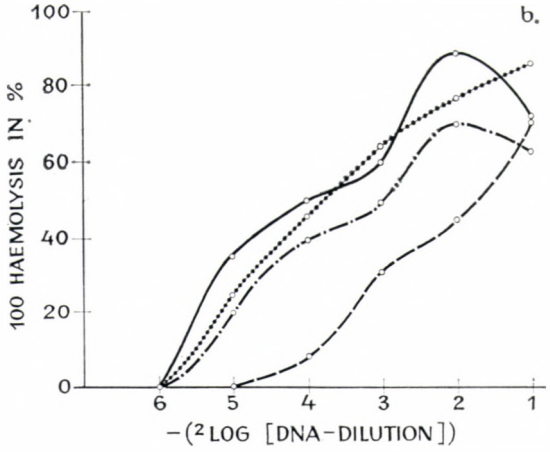

Fig. 3b. Complement fixation by preparation *A* and 1 : 100 diluted anti-friburgensis serum. Designations are as in Fig. 2

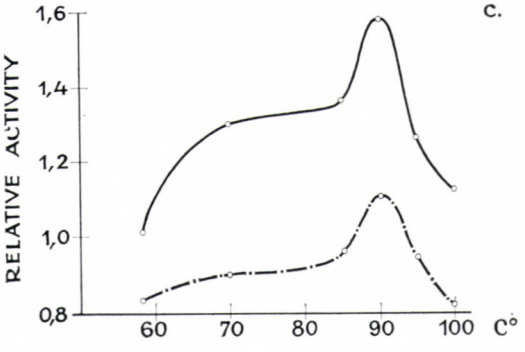

Fig. 3c. Complement fixation by heated preparation *A*. Designations are as in Fig. 2

Phenol and chloroform-treated *M. smegmatis* preparation B contained approximately 15 per cent RNA and less than 1 per cent protein.

Complement fixation by this preparation (Fig. 4*b*) was the most effectively decreased by DNase; the activity decreased very slightly after carboxypeptidase decomposition, while trypsin, RNase and lysozyme exerted no influence whatever.

The relative activity of the heated preparation is presented in Fig. 4*c*. Between 40 and 60° C, two activity maxima, between 90 and 96° C, three maxima and three minima, can be observed (Fig. 5). It is noteworthy that the activ-

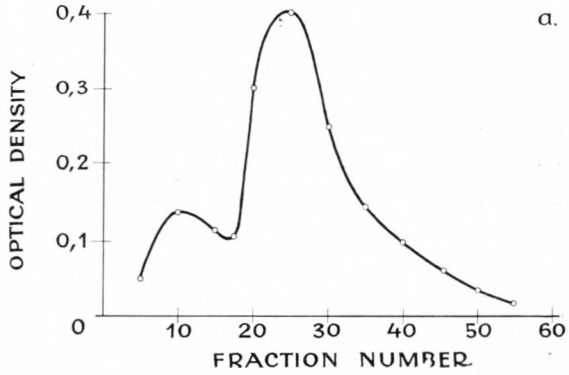

Fig. 4a. Chromatography of *M. smegmatis* preparation *B*

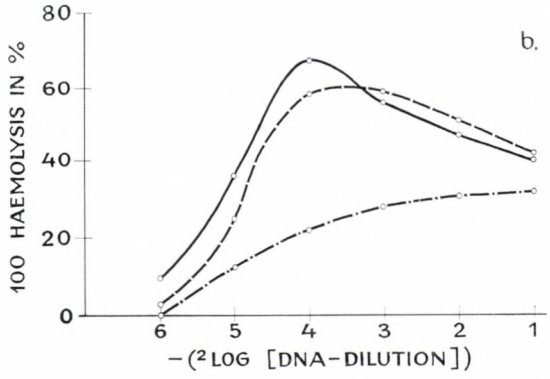

Fig. 4b. Complement fixation by preparation *B* and 1 : 100 diluted anti-friburgensis serum

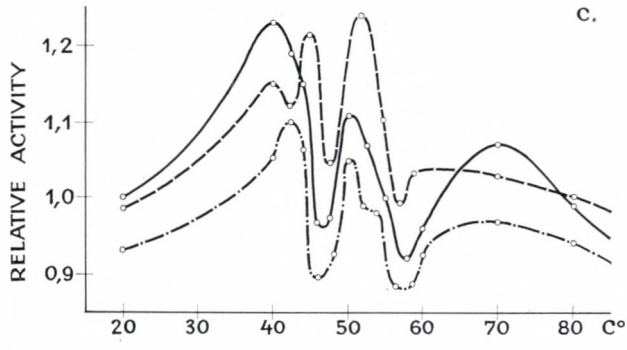

Fig. 4c. Complement fixation by heated preparation *B*. Designations are as shown in Fig. 2

ity of the carboxypeptidase-treated and heated preparation was higher at certain temperatures than that of the untreated material. As compared to the control, between 40 and 60° C the maxima and minima showed a slight lateral

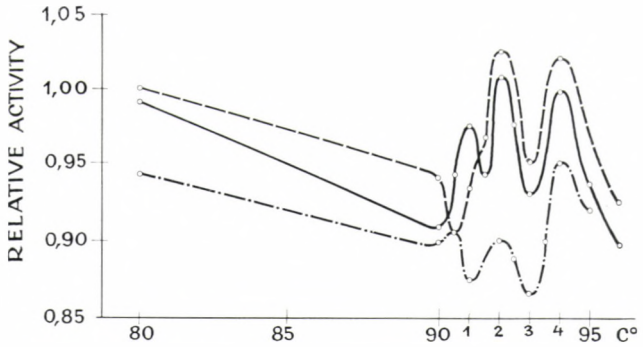

Fig. 5. Complement fixation by preparation *B* after gradual heating from 90° to 94° C in steps of 0.2° C

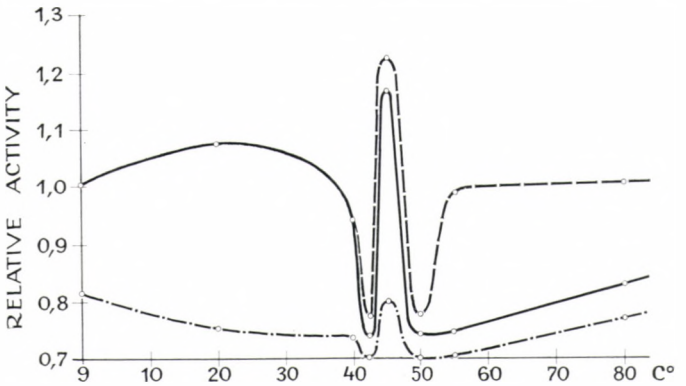

Fig. 6. Complement fixation by chromatographed *M. smegmatis* DNA (preparation *C*) after heating at various temperatures between 0 and 80°C

shift. At 92 or 94°C they corresponded to the peaks observed in the control.

Complement fixation by this preparation after DNase decomposition and heating was weaker than by the untreated control. The range of the peak values for the DNase-treated preparation corresponded to the peaks for the control.

The chromatographed *M. smegmatis* DNA (preparation C) showed a slight increase in activity when heated to 20° C. The increase was considerable at 46°C; between 90 and 94°C two activity maxima and one minimum were shown (Figs. 6 and 7). After DNase treatment and heating there was a considerable

loss in activity. Except for 20°C, the range of peaks also corresponded to that observed with the control.

After carboxypeptidase digestion and heating, between 0 and 20°C the preparation exerted a serological activity similar to that of the control. At 42°C or over the treated material showed a higher activity.

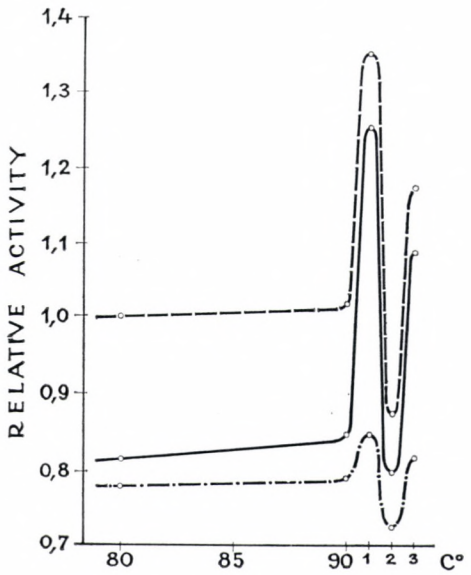

Fig. 7. Complement fixation by chromatographed *M. smegmatis* DNA (preparation *C*) after heating at various temperatures between 80 and 93° C

It should be noted again that heating of the digested preparations was carried out in the presence of the corresponding enzyme; the influence of enzyme proteins will be discussed below.

Discussion

In preparations containing high amounts of contaminating substances the serological activity of DNA cannot be separated from that of proteins, polypeptides, polysaccharides and perhaps RNA.* As confirmed by enzymic digestion, the purification procedure effectively reduces the activity of contaminating substances. When treated with DNase, purified DNA preparations show a decrease in serological activity. This indicates that DNA is an active component of the serological reaction.

* While this paper was being composed E. KÁLMÁN, F. ANTONI and F. VÁRTERÉSZ have published a study on the serological activity of ribonucleic acids (Acta microbiol. Acad. Sci. hung. **9**, 341, 1962/63).

The carboxypeptidase sensitive component is not removed by chloroform treatment. Purification with phenol, however, removes that factor.

As it has been reported [2], the serological activity of the preparations is not completely eliminated by DNase digestion, even when chromatographically separated substance is used. This finding indicates the presence of contaminating substances undetectable by the chemical and enzymic methods used. The possible explanation of the finding has been discussed previously [2].

STOLLAR and LEVINE [12] showed a correlation between the relative complement fixation by heated DNA antigens and the relative optical density measured after the heating of DNA (MARMUR and DOTY [13, 14]).

Although the procedures used for the isolation and heat-treatment of our DNA preparations and our complement fixation method differed from the technique applied by STOLLAR and LEVINE, the considerations advanced by these authors should have been valid for our preparations. It was, however, found that, even with chromatographically purified DNA, the curve representing the antigenic activity of heated antigens was far from being similar to the optical density curve. The measured alterations, as follows from the experiments, were not due to contaminating substances, but to the DNA itself.

The amount of complement fixed by heated DNA preparations varies with the temperature of heating.

It may be assumed that heating causes an alteration in the "shape" of the antigen and, consequently, in the "surface" accessible to the antibody. The term "surface" is used here as generally accepted for synthetic chain polymers [22].

The reasons for shape and surface alterations may be outlined as follows.

Heating causes a break in the hydrogen bonds between the guanine-cytosine and adenine-thymine couple; thus the surface of the antigen increases and active groups are liberated [12]. The break may occur simultaneously along the whole chain [15], or only at certain parts of the molecule [16].

Another reason for surface alteration may be that DNA molecules in saline are not stiff straight strands [17], but some coilings may occur. Measurements carried out by CERF [18], REICHMANN [19] and ROWEN [20] also indicate the deformability of DNA molecules.

Inhibition due to steric conditions prevents configurational changes in such complex molecules [21]. However, at energy transfer these steric hindrances are partly overcome and the whole molecule may appear in an altered configuration; in other words, a conversion to a higher energy state may occur. The rapid cooling after energy transfer may stabilize the higher energy state.

As regards surface alterations in DNA molecules, a hypothesis conforming with our knowledge of structural data of colloids, plastics and DNA, may be advanced for the antigenic activity changes due to heating.

At low temperatures the position of the two chains is stabilized by intact guanine-cytosine and adenine-thymine linkages. The surface accessible to the antibody is determined by a secondary or tertiary coiling [23] or a chance winding of the helix. Considering that the peak values of serological activity in various mycobacterial DNA preparations have been found at an almost identical temperature range, a chance winding seems unlikely.

At rising temperatures the breaking of H bonds also contribute to surface changes in the antigen. A further complication is that, owing to the coiling of liberated chains or chain particles [16], interactions may occur with adjacent molecules or among the incriminated particles within the same molecule [24, 25]. The single-stranded chains and their particles are more liable to coiling than the helix consisting of two chains.

This latter hypothesis seems to be supported by the fact that the serological activity of chromatographed and heated DNA exhibited more considerable changes within a relatively narrow temperature range in the vicinity of the melting point than between 0 and 60° C. In addition, with single-stranded forms rotation may occur around —C—O—P—O—C— linkages, when certain groups become inaccessible to antibodies.

STOLLAR and GROSSMANN [26] have shown that when a melted and quickly cooled DNA solution was re-heated, an optical density maximum occurred at 46° C. Although this maximum stands close to one of our peaks observed between 40 and 60° C, the optical and serological peak values cannot be attributed to a common factor, because in order to induce an optical maximum, the DNA must be denatured previously. With our preparations, at least with the chromatographed DNA, the possibility of denaturation could be excluded.

After carboxypeptidase digestion and heating at certain temperatures, the serological activity of DNA preparations increased. This may have been due to the stabilizing effect of proteins. Studies on this problem will be reported in a subsequent paper.

Acknowledgement. The author is indebted to Mr. A. TOMCSÁNYI for chromatographic examinations, and Miss SAROLTA HORVÁTH for skilled technical assistance.

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EXAMINATION OF THE CYTOPATHIC EFFECT OF ADENOVIRUSES BY IMMUNOFLUORESCENCE

By

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Summary. Types 3 and 5 of adenovirus characterized by different types of cytopathic effect have been studied by immunofluorescence in primary human amnion cell cultures. It has been shown that the structures appearing in the cell after virus infection had the same antigenic character as the virus. The data obtained suggested that virus (or viral antigens) are formed as early as in 7 to 10 hours following infection. An asynchronism in the onset of virus multiplication was also observable. In the final stage, however, all cells exhibited specific fluorescence.

The morphological changes produced by adenoviruses in primary human amniotic cell cultures are well-known to exhibit a wide variety of patterns. Nevertheless, two main types of cytopathic effect may be distinguished and the adenoviruses grouped accordingly. The most conspicuous characteristics being those exhibited by type 3 and 5 adenovirus, thus the two morphological types were designed as type 3 and 5 degenerations, respectively. Details of these studies have been published [1]; we only refer here to the fact that both nuclear and cytoplasmic changes are different in the two types of cytopathic alteration. Particularly conspicuous is the difference in the arrangement of the degenerated cells. In type 3 degeneration the cells form a network-like pattern, while in type 5 degeneration the cells are single or form small groups. In the present study the localisation of virus synthesis has been examined by following the development of nuclear changes by immunofluorescence. The time sequence of the formation of virus-specific particles was also studied.

Materials and methods

Viruses. The 3/4 strain of type 3, and the 5/5 strain of type 5 adenovirus was used throughout [1]. Both viruses were propagated in Detroit-6 cell cultures. Titrations were performed in primary human amnion cells.

Tissue cultures. Primary human amnion cell monolayers were obtained by cultivating the cells on 6×18 mm cover slips immersed in test tubes. Into each tube was poured 1 ml cell suspension, containing 3×10^5 cells per ml. The medium consisted of Hanks' solution containing 5 per cent lactalbumin hydrolysate and 20 per cent human serum. The maintenance medium contained 5 per cent calf serum and 0.25 per cent lactalbumin hydrolysate in Hanks' solution. Antibiotics were added to both types of media.

Immune sera. Type specific sera were prepared in rabbits [2]. The tissue antibodies were absorbed by HeLa cells used for virus propagation.

Staining. The tissue cultures were stained by the immunofluorescent method first described by COONS and KAPLAN [3]. Virus-specific antigens were detected indirectly using fluoresceine isocyanate coupled anti-rabbit-globulin. The dye used for conjugation was dissolved in carbonate-bicarbonate buffer (pH 9) instead of dry acetone. The concentration of the stain was usually 10 mg per ml.

Experimental procedure. Tissue cultures were washed with Hanks' solution and infected with 100 cytopathogenic units (CPU) per culture. After 30 minutes of adsorption 1 ml of maintenance medium was added to each tube. The infected cultures were incubated at 37° C, and at intervals groups of 3 tubes were removed and fixed in methanol for 20 minutes. The points of time of sampling were, 1, 2, 4, 8, 16 hours, 1, 2, 4, 5, 7, 8, 12 and 14 days, following the infection. Uninfected cultures treated in the same way served as controls.

The cover slips with the cell monolayers were removed from the tubes for staining. Two cover slips each from the control and infected cultures were stained simultaneously in the usual way. Similar cover slip preparations were used as serum controls. These, however, were treated with a heterologous rabbit immune serum.

For microscopic examination the cultures were mounted on a plexiglass support provided with a hole to permit free passage of ultraviolet light. The apparatus used was a Leitz Ortholux II type fluorescent microscope. The usual magnification was 250fold in blue light.

Results

The uninfected control cultures exhibited the usual pattern of normal human amnion cell cultures. A moderate greenish autofluorescence was generally present. The contours of the cells could be clearly distinguished while no nucleoid could be seen (Fig. 1).

Cultures stained 1 or 2 hours after the infection exhibited a pattern identical with the controls. After 8 hours' incubation small, greenish fluorescent granules were demonstrable in certain nuclei. In the 16th to 24th hours following the infection these granules became more conspicuous, more numerous and in certain cells the formation of a fluorescent ring could be observed. In these cells the granules were already arranged in an inclusion-like pattern.

On further incubation in some cells the whole nucleus became fluorescent. Except for this the cultures did not exhibit any visible pathologic change and the cells formed a confluent layer. In 4 to 5 days old cultures parallel to a numerical increase of cells with nuclear fluorescence the morphology of the cells began to change. The green fluorescence became more and more bright and the confluent character of the monolayer began to disappear. On the 7th to 8th day there were practically no normal cells present and the arrangement typical of type 3 or type 5 degeneration started to develop. With the shrinkage of the cytoplasm and the nucleus, the nuclear fluorescence turned from green to a yellowish-green (Figs. 2 and 3). This type of fluorescence was present in every nucleus in aged (12—14 days old) infected cultures. The cells were shrunk and in many of them the protoplasm was but a thin layer around the nucleus (Figs. 4 and 5).

Infected cultures treated with heterologous serum exhibited from the 4th day on, parallel with the progress of the cytopathic changes, a successive,

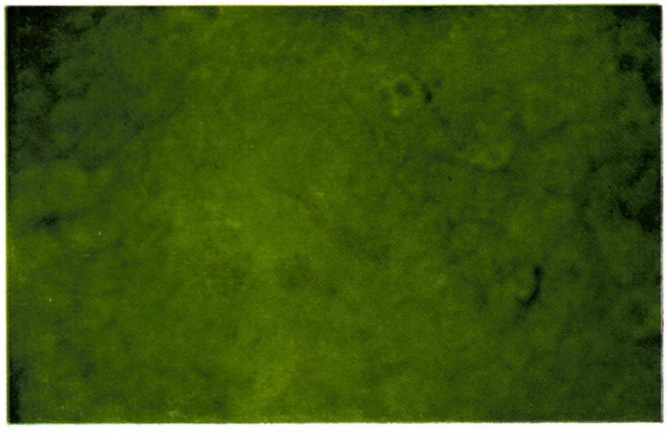

Fig. 1. Uninfected primary human amniotic cell cultures. Magnification $\times 600$

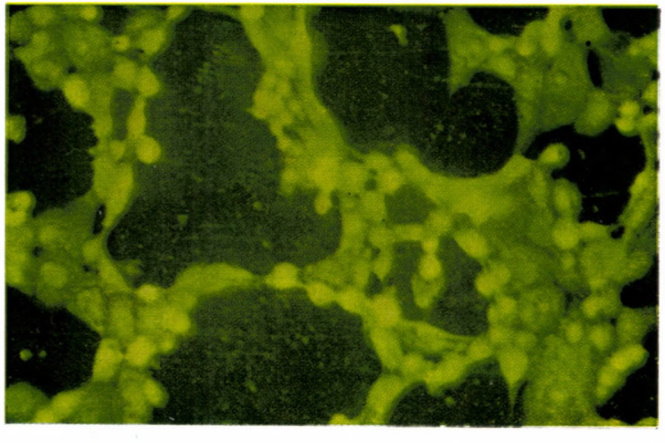

Fig. 2. Culture infected with 3/4 strain of adenovirus and treated with homologous serum, 8 days after infection. Magnification $\times$ 600

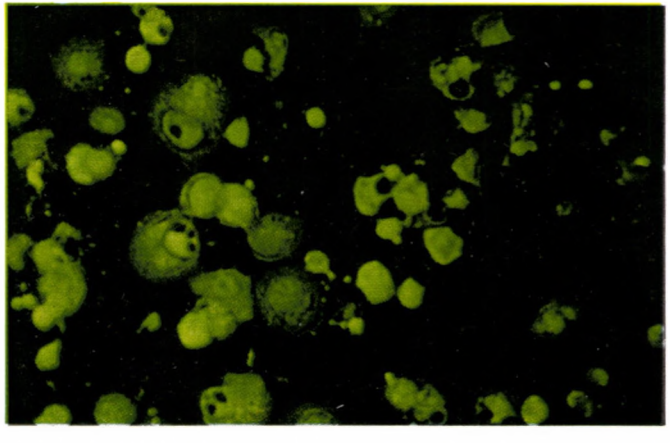

Fig. 3. Culture infected with 5/5 strain of adenovirus and treated with homologous serum, 7 days after infection. Magnification $\times 600$

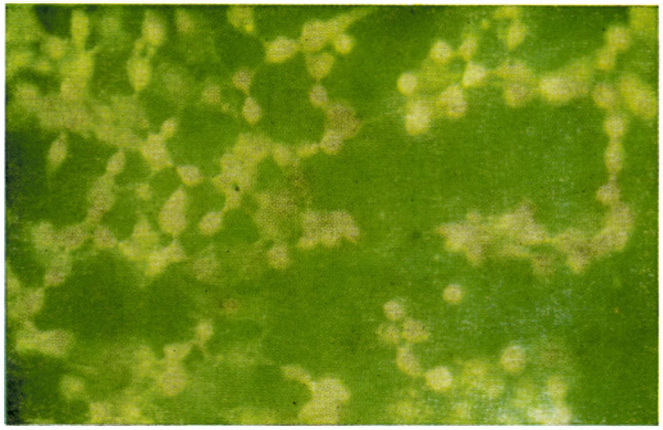

Fig. 4. Culture infected with 3/4 strain of adenovirus and treated with homologous serum, 14 days after infection. Magnification × 600

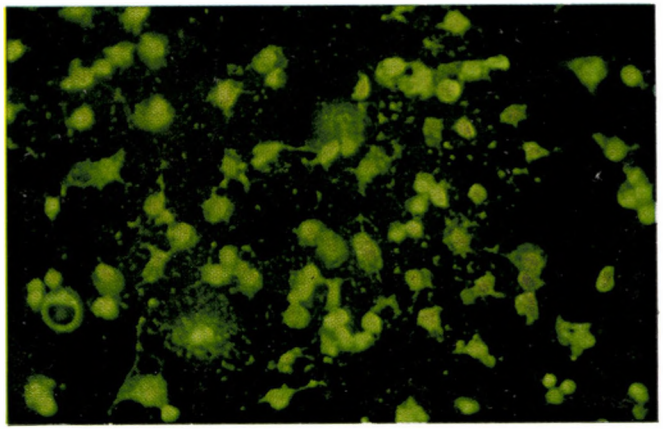

Fig. 5. Culture infected with 5/5 strain of adenovirus and treated with homologous serum, 12 days after infection. Magnification × 600

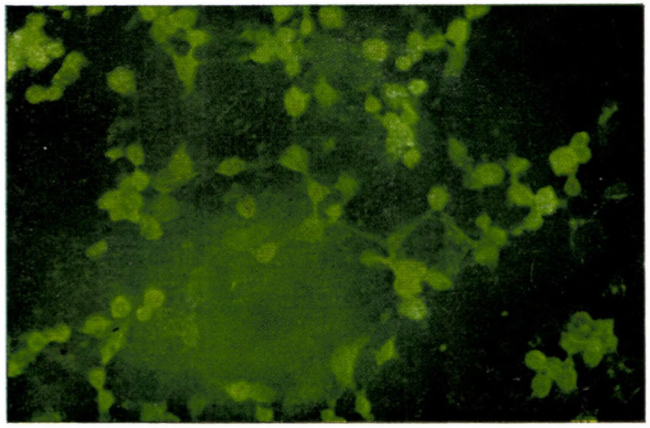

Fig. 6. Culture infected with 3/4 strain of adenovirus and treated with heterologous serum, 14 days after infection. Magnification × 600

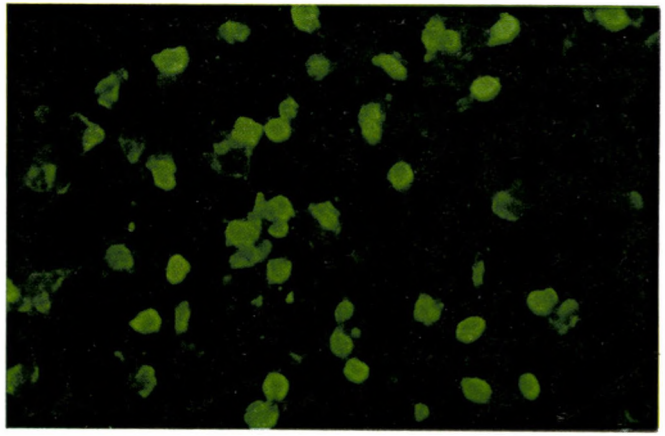

Fig. 7. Culture infected with 5/5 strain of adenovirus and treated with heterologous serum, 12 days after infection. Magnification × 600

though slight, increase of autofluorescence. On the 14th day the fluorescence began to change from greyish-green to pure green (Fig. 6 and 7).

Discussion

The characteristics of the cytopathic effect produced by adenoviruses have been studied by a number of authors [1, 4 to 8]. The results permitted a broad analysis of the relations between viral effect and pathomorphology [4, 9 to 11]. Cultures inoculated with killed virus failed to damage the ceils. The positive Feulgen reaction suggested an accumulation of desoxyribonucleic acid in the nuclei of virus-infected cells. This observation did not, however, exclude the possibility that the different structures found in the nuclei might contain virus, a supposition supported by the observations of PEREIRA *et al.* [4] and BOYER *et al.* [15].

Our present findings are in agreement with those mentioned above and give some further information about the development of the different morphological units appearing parallel with the cellular degeneration. In human amniotic cell cultures the light microscope reveals the first signs of virus infection after 48 hours. By immunofluorescence the appearence of some green corpuscles may be observed after 4 to 8 hours already and subsequently the characteristic fluorescence extended to the whole nucleus. Thus it seems, that the virus or virus-specific antigens appeared in a few hours following infection, while in the uninfected controls no changes were detectable. As to the early onset of virus synthesis, we refer to the studies of GREEN [12, 13] on type 2 adenovirus in KB cells. This author demonstrated a complete inhibition of virus synthesis after having added 5-fluoro-deoxyuridine to the infected cultures at any time during the first 7 hours. After 7 hours inhibitory effect was no longer demonstrable. Thus he concluded that viral DNA synthesis started after 7 hours following infection. Similar observations were made also by KJELLÉN [14]. All these results suggest that virus synthesis starts soon after the infection and in 7 to 10 hours it reaches a level demonstrable not only by biochemical methods but also by immunofluorescence.

Tissues treated with heterologous serum did not exhibit fluorescence. Nevertheless, the greyish-green autofluorescence turned into a clear green from the 4th day of incubation. This, however, did not interfere with evaluation, as the difference between the uninfected controls and the infected cultures treated with heterologous or homologous serum was quite definite. Apparently, the soluble antigens did not disturb the reactions under our experimental conditions.

The results presented have shown that in this particular case the virus was produced in the nucleus. The onset of virus replication was not synchronous.

Thus 8 to 16 hours after the infection the incidence of morphological changes was rather scarce. Later on the pattern became more and more heterogeneous and a variety of cells in different phases of cytopathic changes were simultaneously present. In the final stage, however, every cell exhibited a yellowish-green fluorescence.

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ENTERITIS ASSOCIATED WITH AN UNUSUAL BIOCHEMICAL VARIANT OF ESCHERICHIA COLI SEROTYPE O26:B6:H11

By

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Summary. An outbreak characterized by enteric and respiratory symptoms occurred in January, 1963, in the day nursery of *Sirok* (county *Heves)*. From the faeces of an affected infant and a healthy adult, quick-lactose-fermenting *E. coli* strains belonging to serotype O26:B6:H— were isolated. The faeces of two sick infants yielded biochemical variants of *E. coli* serotype O26:B6:H11. These cultures fermented lactose between 6 and 10 days. In contrast to the former strains, the slow-lactose-fermenting organisms grew readily, and formed shigellaform colonies, on desoxycholate citrate agar. Haemagglutination with patients' sera revealed with one exception, no significant agglutinin response to the supposed causative agent.

The pathogenicity of *E. coli* O26:B6 was first suggested by ØRSKOV in 1951 [10], who demonstrated the frequent occurrence of this type in sporadic cases of infantile enteritis. Since ØRSKOV's observation a number of authors in various parts of the world have confirmed the aetiological role of that organism in both sporadic cases and outbreaks of infantile enteritis [1—3, 5, 8—11, 14]. The organism is generally regarded as one of the most commonly occurring pathogenic *E. coli* strains.

Our material was obtained from a nursery outbreak in *Sirok* (county *Heves)*, during January, 1963. Out of the 16 infants kept in the day nursery, 10 were affected. The outbreak was characterized by a sudden onset, acute respiratory symptoms, vomiting and diarrhoea, temperatures of 38—40° C, and dehydration. One infant died after 1 $^1/_2$ days, during transportation to hospital. At autopsy cerebral congestion was found. Other affected infants were admitted to hospital and were treated with antibiotics, sulphonamides, infusions and vitamins. Their condition improved in 7 to 8 days. Because of tonsillitis, otitis and other complications, complete recovery ensued generally after 14 days.

Soon after the appearance of the first infections parallel faecal samples from 13 infants and 2 nurses were examined in the Department of Bacteriology, State Institute of Hygiene, and in the laboratory of the Regional Public Health Station, Eger. For virological examination the same specimens were sent to the Department of Virology, State Institute of Hygiene.

During hospital treatment the patients were subjected to further bacteriological examinations.

Seven days after the fatal case, the faeces of the died infant's father was also examined.

The *E. coli* O26:B6 strains were isolated from eosin-methylene blue agar by the slide agglutination method. In the serological analysis, strains F 41, O26:K60(B6) : H— and Su 4321/41, O13:K11L:H11, received from the State Serum Institute, Copenhagen, were used as controls. Preparation and absorption of immune sera and determination of antigens were performed as described by KAUFFMANN [7] and EWING *et al.* [4], with the exception that all tube agglutination tests were read after 18—20 hours' incubation at 37° C.

Biochemical reactions were carried out as described in the International Bulletin of Bacteriological Nomenclature and Taxonomy [6]. Antibiotic sensitivity was tested by use of Biotest paper discs (Institute for Serobacterial Production and Research "Human", Budapest).

Antibody content of the patients' serum was examined by the haemagglutination method. The reaction was carried out with 2.5 per cent sheep erythrocyte suspension sensitized as described by NETER *et al.* [9] with the supernatant of the boiled suspension of one of the slow-lactose-fermenting *E. coli* O26 : B6 strains. Dilution of sera and determination of haemagglutinin titres were made by TAKÁTSY's spiral loop method [13]. All sera were checked for normal sheep haemagglutinin titre.

Bacteriological examinations

Of the 16 persons examined bacteriologically, 4 yielded positive faecal cultures for *E. coli* O26 : B6. Three strains were isolated from the affected infants and one healthy adult. Cultural examinations performed at the same time in the State Institute of Hygiene and in the Regional Public Health Laboratory yielded similar results. The positive samples were taken in the nursery at the initial stage of the disease, before the administration of antibiotics. Multiple bacteriological control examinations performed during hospital treatment gave negative results. The faeces of the two nurses employed in the nursery were also negative. *E. coli* O26 : B6 was, however, isolated 7 days after the onset of the outbreak from the faeces of the died infant's father. Virological examination of the faecal samples was uniformly negative.

From one infant and from the died infant's father, quick-lactose-fermenting, from two infants late-lactose-fermenting, *E. coli* O26 : B6 strains were cultured. Of the latter, strain 1173 was used in the antigenic analysis.

As shown in Table I, the strains were biochemically typical *E. coli* cultures. The strain isolated from the adult person differed from the slow-lactose-fermenters in splitting dulcitol and raffinose promptly, and sucrose after 4 days.

The slow-lactose-fermenting cultures grew readily on desoxycholate citrate agar and produced colonies resembling those of shigellae. In contrast, the growth

Table I

Biochemical behaviour of slow-lactose-fermenting E. coli strain 1173

Glucose	++	Raffinose	—
Mannitol	++	Sucrose	—
Lactose	+$^{6-10}$	Urea	—
Maltose	+	Methyl red	+
Rhamnose	+	Voges-Proskauer	—
Sorbitol	+	Indole	+
Xylose	+	Malonate	—
Salicin	+^{5}	KCN	—
Dulcitol	—	Ammonium citrate	—
Adonitol	—	H_2S	—
Inositol	—	Tryptophan deaminase	—

++ acid and gas production
+ acid production or positive reaction
— negative reaction

Table II

Analysis of O and K antigens of slow-lactose-fermenting strain 1173

Antigens	OK serum, F 41		OK serum, 1173		Unabsorbed O serum	
	Unabsorbed	Absorbed by strain 1173 (living)	Unabsorbed	Absorbed by strain F 41 (living)	F 41	1173
F 41 living	5 120	0	1 280	0	0	0
1173 living	1 280	0	1 280	0	0	0
F 41 1 hr 100° C	10 240	0	10 240	0	40 960	40 960
1173 1 hr 100° C	10 240	0	10 240	0	40 960	40 960

Table III

Analysis of the H antigen of slow-lactose-fermenting strain 1173

Antigens*	H serum, Su 4321/41		H serum, 1173	
	Unabsorbed	Absorbed by strain 1173	Unabsorbed	Absorbd by strain Su 4321/41
Su 4321/41	409 600	0	409 600	160**
1173	204 800	0	409 600	0

* Formalized swarm agar cultures
** O-type agglutination

of our quick-lactose-fermenting strains, as well as of type strain F 41, was inhibited by desoxycholate citrate medium in a degree typical of *E. coli* cultures.

As indicated by the agglutination and absorption tests shown in Tables II and III, the antigenic structure of the slow-lactose-fermenting strain corresponded to O26:K60(B6):H11. Cross absorption tests revealed that the O and K antigens of strain 1173 were identical with those of strain F 41, while its flagellar antigen was identical with the H antigen of strain Su 4321/41. The antigenic structure of the quick-lactose-fermenting strain was O26:K60(B6):H—. In other words, the culture was a non-motile variant serologically corresponding to type strain F 41.

As to drug sensitivity, the slow-lactose-fermenting strains were sensitive to chloramphenicol, streptomycin, neomycin and polymyxin B, resistant to penicillin, tetracyclines, erythromycin and sulphonamides. The quick-lactose-fermenting organisms differed from the former cultures in their tetracycline sensitivity. One of the latter cultures was sensitive, the other was moderately sensitive to streptomycin.

None of the strains caused experimental keratoconjunctivitis in guinea pigs [12].

The antibody titre in 12 blood samples was examined by the haemagglutination technique. Serum samples of 40 healthy individuals sent in for Wassermann reaction and of 38 patients sent for Widal reaction were examined as controls. One infant's serum agglutinated erythrocytes sensitized with one of the slow-lactose-fermenting *E. coli* strain in a titre of 1 : 128. The serum sample taken from the same infant 6 days later showed, however, only an 1 : 4 titre. Apart from this case, there was no significant difference in titre between the control and the patient sera.

Discussion

The enteric symptoms observed in the outbreak have been ascribed to the *E. coli* 26:B6 strains isolated from the faeces of the patients. The small number of positive results may have been due to the fact that, under the impression of the rapid death in the first case, the affected infants were transferred to hospital immediately after the appearance of the first symptoms of illness. In the hospital antibiotic treatment was immediately started. From the hospitalized infants faecal samples were obtained generally only some days after admission. As in every patient respiratory infection was evident, and in some cases even predominating, it may be assumed that this condition, as a disposing factor, had promoted the manifestation of enteric symptoms. The causative role of *E. coli* O26:B6 has not been confirmed, neither excluded, by the negative haemagglutination results, it being well known that in a large

number of enteric infections no increase in antibody titre can be demonstrated.

It is remarkable that in the outbreak two different *E. coli* O26:B6 organisms, but no other known pathogenic bacteria were encountered. The two kinds of *E. coli* O26:B6 cultures were undoubtedly distinct types, because, in addition that one of them was a non-flagellated mutant, they differed in at least 4 biochemical reactions, in antibiotic sensitivity pattern, and in cultural properties.

As in the literature available only quick-lactose-fermenting *E. coli* O26:B6 strains have been described, the present findings seemed to merit a certain interest.

The shigellaform growth on desoxycholate citrate agar of our slow-lactose-fermenting cultures is uncommon with other O26:B6 strains. As to this property, our strains remind of *E. coli* O124:B17, the known aetiological agent of dysenteriform sporadic infections and outbreaks affecting adults as well as children. While, however, O124 : B17 strains produce experimental keratoconjunctivitis in guinea pigs, our slow-lactose-fermenting cultures failed to give this reaction.

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EFFECT OF CYSTEINE ON LOCAL SHWARTZMAN PHENOMENON

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Summary. Simultaneous administration of the provoking dose of endotoxin and intravenous cysteine to rabbits prepared for local Shwartzman reaction has been found to inhibit thrombopenia and enhance the decrease in the number of circulating leukocytes. As compared to the control animals, cysteine treatment promoted the quantitative Shwartzman phenomenon both in intensity and duration.

Experiments *in vitro* have shown that in platelet-rich rabbit plasma cysteine and some thiol compounds inhibit the endotoxin-induced platelet aggregation and the release of histamine and serotonin [1]. As in the pathogenesis of the Shwartzman phenomenon a considerable importance is attributed to platelets, it was of interest to examine whether cysteine influenced the platelet count and the development of local Shwartzman phenomenon *in vivo*.

Materials and methods

Rabbits of both sexes weighing 2 to 3 kg were used. Endotoxin was prepared from *E. coli* by the method of BOIVIN *et al.* [2]. Preparation of 19 rabbits was carried out by injecting 0.1 ml aliquots of various dilutions of the endotoxin stock solution intradermally into the shaved dorsal skin, according to the method of KESZTYŰS *et al.* This technique allows a semiquantitative estimation of the results [3]. The provoking dose of 1 mg/kg endotoxin was given intravenously 24 hours after the preparatory injection.

Simultaneously with the provoking dose, 8 rabbits were given 200 mg/kg cysteine HCl intravenously. Cysteine was previously neutralized with NaOH and, to avoid alkalization, a small amount of tris buffer was mixed to the solution. The 8 control animals received equal volumes of NaCl solution equivalent in molarity to cysteine. Three rabbits injected with cysteine received no endotoxin. After the injections platelet and leukocyte counts were made at various intervals. The local Shwartzman reaction was read 3 and 24 hours after the administration of the provoking dose.

For platelet counting, 0.1 ml blood was withdrawn from the auricular vein into polyethylene tubes containing 0.9 ml 3.8 per cent sodium citrate. After the erythrocytes had been removed by centrifugation, the platelets were counted directly in Bürker chambers. Leukocyte count was determined by the usual technique.

Results

Fig. 1 shows the result of platelet counting. It is seen that, in the control rabbits after 5 minutes the average count decreased to 45 per cent of the original. The initial count was not reached in 60 minutes. In cysteine-treated rabbits the decrease was considerably less, and the count became normal after 30 minutes.

In contrast, Fig. 2 shows that the number of circulating leukocytes decreased considerably in both groups. Thus, endotoxin-induced leukopenia was promoted rather than inhibited by cysteine.

Fig. 3 presents the result of the quantitative Shwartzman reaction. At the 3 hour reading definitely increased local reactions were observed in the

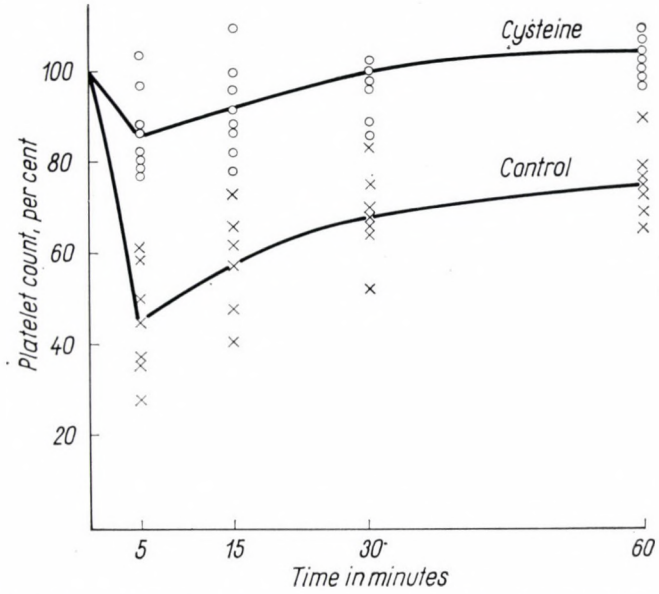

Fig. 1. Platelet count in control and cysteine-treated Shwartzman rabbits

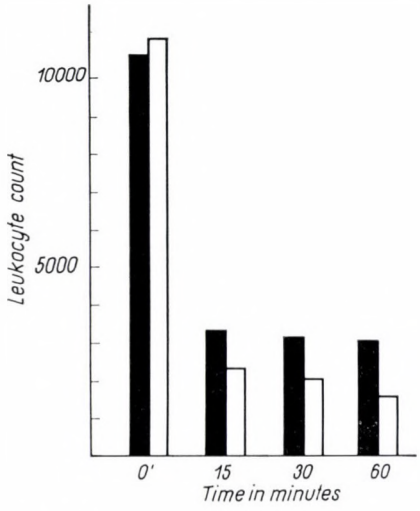

Fig. 2. Leukocyte count in control and cysteine-treated Shwartzman rabbits

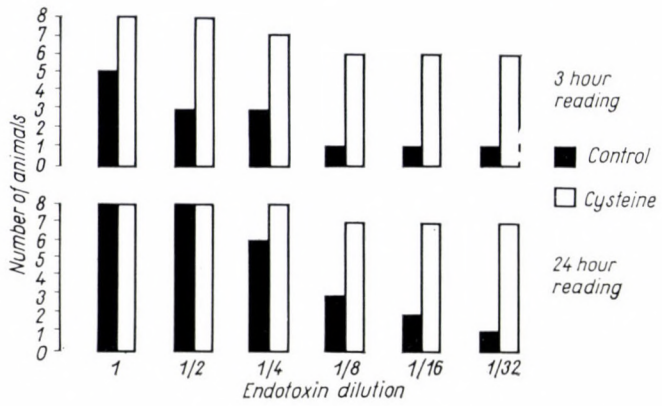

Fig. 3. Local Shwartzman reaction in control and cysteine-treated rabbits

cysteine-treated animals. After 24 hours the difference was less definite between the control group and the one treated with low dilutions of endotoxin, while the difference remained unaltered between the controls and the animals treated with high endotoxin dilutions. Accordingly, cysteine enhanced the local Shwartzman phenomenon both in intensity and duration. In control animals treated with cysteine only, significant changes in platelet and leukocyte counts and the Shwartzman reaction were not observed.

Discussion

It has been shown that single intravenous endotoxin injections give rise to thrombopenia and leukopenia [4, 5]. From platelets and platelet aggregates retained in the small vessels biologically active substances are released. The leukopenia is due primarily to an adhesion of leukocytes to the inner wall of small vessels [4]. Thrombi consisting of platelets and leukocytes in these vessels are the most characteristic histological finding in local and generalized Shwartzman phenomenon. The rupture of vessels is preceded by the formation of such thrombi [4]. The appearance of thrombi, shortened blood coagulation time [7—11], deposition of fibrinoid [7, 12] and the simultaneous decrease of the fibrinogen level [7] indicate an involvement of the coagulation system in the reaction. This consideration is supported by the findings that anticoagulants as heparin [13], or sodium warfarin (an oxycoumarin derivative), inhibit the Shwartzman reaction. Des Prez, Horowitz and Hook showed that endotoxin caused an agglutination of platelets in platelet-rich rabbit plasma *in vitro*, and a subsequent release of platelet factor 3 in addition to serotonin and bactericidins. Platelet factor 3 is responsible for the shortened coagulation time. This means that the coagulation disturbance is associated with the platelets, because in platelet-poor plasma endotoxin is incapable of decreasing the coagulation time [10, 11].

While in the opinion of the mentioned authors the coagulation system and thus the platelets play a primary part in the mechanism of the Shwartzman reaction, other experiments indicate the important role of leukocytes. The Shwartzman reaction cannot be provoked in animals with leukopenia induced by N-mustard, benzene [15] or X-rays [17]. In contrast, any substance capable of provoking the Shwartzman reaction (antigen-antibody, glycogen, dextran, kaolin) cause thrombopenia and leukopenia [4]. The platelet and leukocyte reaction to endotoxin indicates that the thrombopenia and leukopenia associated with the Shwartzman phenomenon can be attributed to similar mechanisms.

In our experiments, when cysteine was applied simultaneously with the provoking dose, the decrease of platelet count was inhibited and the local reaction definitely promoted. In contrast, cysteine had no influence on the changes in leukocyte count. From these data the following conclusions have been drawn.

(i) The fact that cysteine inhibits the development of thrombopenia but not of leukopenia, shows that two different mechanisms are involved.

(ii) As cysteine inhibits the aggregation of platelets *in vivo* as well as *in vitro* but does not affect the development of local reaction, in releasing the Shwartzman phenomenon the production of platelet aggregates seems to be of less importance than the role of leukocytes.

As no histological examinations have been performed, this theory can be considered a tentative consideration only. It may be supposed that, although cysteine inhibits the clumping of platelets, it might enhance some activating processes, which will predominate in the reaction.

(iii) The promoting effect of cysteine on leukopenia and the Shwartzman phenomenon points to the importance of leukocytes. The highly injurious effect exerted by leukocytic and mononuclear infiltration of the vessel walls at the site of the preparatory injection [4, 15, 17, 18] and aerobic intracellular glycolysis [16] constitute a disposing factor for the development of leukocyte-platelet thrombi. The high proteolytic enzyme content of leukocytes probably also contributes to the further destruction of the injured vascular walls, and thus enhances the development of haemorrhagic necrosis. The activity of leukocytic proteases is increased by the acid reaction due to aerobic glycolysis and the accumulation of anoxybiotic products. In the opinion of THOMAS and STETSON, SH-proteases are primarily responsible for the destruction of vascular walls, because intradermally given papain, cysteine and BAL are suitable preparatory substances against endotoxin given intravenously one or two hours previously. Trypsin and other reducing agents are ineffective in this respect [16]. Accordingly, the observed promoting effect on the Shwartzman phenomenon of intravenously given cysteine may be explained by the activation of certain leukocytic SH-proteases.

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NEW OBSERVATIONS ON HYDROGEN SULPHIDE PRODUCTION BY ENTERIC BACTERIA

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Summary. (i) Apart from some exceptions all of the enteric bacteria are able to produce H_2S from cysteine or cystine. Methionine is unsuitable as a substrate for H_2S production.

(ii) The positivity of the H_2S test employed in systematic genus differentiation depends upon the ability of certain organisms belonging to the positive genera to form H_2S from sulphates. Therefore, the result of routine testing is the consequence of the sulphate contamination of peptones.

(iii) The use of a cysteine-supplemented medium would decrease or eliminate the differential diagnostic value of the H_2S reaction.

(iv) Hypersensitive lead acetate paper strips indicating diagnostically unimportant traces of H_2S, should also be avoided. According to the present experiments, the indicator of choice is $(NH_4)_2Fe(SO_4)_2$ or $FeCl_2$.

(v) For the standard H_2S test a medium containing sulphate-free peptone, $Na_2S_2O_3$ as a sulphur source, and $(NH_4)_2Fe(SO_4)_2$ as an indicator, has been devised.

Detection of H_2S production is one of the earliest method used for the identification of bacteria (Orlowski, 1895). However, the mechanism of, and the ingredients of the medium responsible for, the reaction, have not been entirely elucidated.

The present work was inspired by the contradictory data concerning the H_2S reaction of *P. morganii*. For determining the systematic position of a microorganism, H_2S production is an important criterion. In studying this problem, further questions arose as to the mechanism of H_2S production. Thus it was desirable to determine whether organic or inorganic sulphur compounds were concerned in it, and which of these substances might be regarded as substrates for H_2S production. A further aim of the present work was to elaborate a medium yielding reliable results for differential diagnosis.

Materials and methods

Strains. The indicated numbers of various enteric bacterial strains were examined for H_2S production: *P. morganii*, 100; *E. coli*, 72; Klebsiella, 30; Salmonella, 128; Shigella, 72; Providencia, 29. In addition, one strain was examined from each of the Arizona, Ballerup Bethesda, Hafnia, Cloaca and Serratia genera. The majority of the strains were obtained from the culture collection of this institute. Some freshly isolated cultures were also used. Inoculations were made from 24 hour agar slant cultures.

Media. Preparation of the *basal synthetic medium* was performed by the modified method of Pelczar and Porter [18] as follows.

The basal medium containing 1.0 g Na_2HPO_4, 1.0 g KH_2PO_4, 0.1 g $MgCl_2$, 5.0 g glucose, 5 mg nicotinic amide and 5 mg calcium pantothenate in 1000 ml distilled water, was sterilized

*

by Seitz-filtration. When a solid medium was required, 2 per cent washed agar was added to the basal medium. After steaming without pressure for 1 hour, the medium was filtered and then autoclaved at 120° C for 15 minutes. Supplementary growth factors and amino acids were sterilized by Seitz-filtration and added subsequently to the basal medium. The amino acid requirement of *P. morganii* was examined using 0.1 to 0.25 per cent concentrations of alanine, arginine, asparagine, aspartic acid, cysteine, cysteinic acid, cystine, glutamic acid, glycine, histidine, iso-leucine, leucine, lysine, methionine, norvaline, proline, phenylalanine, threonine, tryptophan, serine, valine and tyrosine.

Peptone-containing liquid or solid media. For the preparation of these media Richter, Bacto (Difco), Proteose (Difco), Witte and Orthana special peptones were used.

The media prepared in the usual manner with various peptones were supplemented with the sulphur-containing amino acids cystine, cysteine and methionine. The filtered amino acids were incorporated at 0.1 per cent concentrations. When solid media were required, to peptone water 2 per cent washed agar was added.

Removal of sulphate from peptones. Sulphates were removed by saturating the solution with 40 per cent $BaCl_2$. After boiling for 30 minutes, the mixture was filtered and dialysed against distilled water at 70° C, for 24 hours. The complete removal of sulphates was checked by the $BaCl_2$ test. Titration of barium excess was performed with $N/10$ or $N/100$ H_2SO_4. From the purified and dialysed peptone the usual peptone water medium was prepared. Aliquots of this medium were supplemented with the above concentrations of cystine, cysteine and methionine, or with 0.04 p. c. Na_2SO_4, Na_2SO_3 or $Na_2SO_2S_3$ as inorganic sulphur sources.

Detection of H_2S production was performed in case of liquid media with strips of filter paper (14 cm by 5 cm) impregnated with a saturated neutral lead acetate solution. The strips were inserted in the tubes so as to hang over the liquid medium. To solid media, 0.08 per cent $(NH_4)_2Fe(SO_4)_2$ or $FeCl_2$ was added as an indicator; lead acetate strips were also used.

Inoculation was carried out with a needle. In liquid synthetic media three subcultures were made and the result of the third inoculation was evaluated.

Paper chromatography of peptones was carried out by the ascending and circular technique. As a solvent, butanol : acetic acid : water (4 : 1 : 5) was used. Development was performed with ninhydrin dissolved in butanol—acetic acid mixture, or, in the case of sulphur-containing amino acids, with KCN—sodium nitroprusside [19]. As a control, a standard amino acid mixture containing each amino acid at 0.1 per cent concentration, was applied. Samples of 5 μl aliquots were deposited on the paper by means of a micropipette.

Results

Experiments on media containing organic sulphur sources. The substrate of H_2S production was studied by adding various sulphur sources to synthetic media containing sulphur-free basal ingredients. As already mentioned, the main problem was the H_2S production by *P. morganii ;* therefore the synthetic medium was composed so as to meet the growth requirements of that organism. It has been observed that, in order to obtain satisfactory growth in the absence of sulphur-containing amino acids, the medium must be supplemented with a complicated battery of amino acids. However, a satisfactory growth was obtained when only one sulphur-containing amino acid was added to the basal medium. Since there was no difference as regards H_2S production between media supplemented with only one sulphur-containing amino acid, the latter type of simple medium was employed in subsequent experiments. The validity of observations made with this medium was supported by the fact that other Enterobacteriaceae genera also grew well on the basal medium enriched with a single sulphur-containing amino acid.

After determining the growth requirements, H_2S production was examined with the paper strip method. While in cysteine or cystine-containing media

P. morganii uniformly exerted a considerable degree of H_2S production, in methionine it gave a negative reaction. From methionine even bacteria known as H_2S-producers (Salmonella, Citrobacter, *P. vulgaris* and *mirabilis)* failed to form H_2S. Accordingly, in synthetic medium only cystine and cysteine are suitable substrates, while methionine cannot be attacked by the enzyme sulphhydrase. Identical results were obtained when solid media containing sulphate-free peptones were provided with the above sulphur-containing amino acids.

Another interesting result of these experiments was that enteric bacteria known as H_2S negative organisms, as members of the Escherichia genus, showed an intensive H_2S production in the presence of cystine and cysteine.

The results are demonstrated in Table I, where only those obtained with sulphate-depleted Richter peptone are given.

Table I

Production of H_2S by stab cultures in agar containing 0.1% cystine and 0.08% $(NH_4)_2 Fe(SO_4)_2$

Strains	No. of strains	H_2S production	
		+	−
E. coli (O type strains)	139	135	4
Klebsiella (K type strains)	30	27*	3
Shigella (collected strains)	72	42*	30
Providencia (O type strains)	29	24*	5
P. morganii (collected strains)	100	93	7
Hafnia (type strain 118)	1	1*	—
Cloaca cloaceae (strain 6279)	1	1	—

* Demonstrable only with lead acetate paper strip

From Table I it is clear that on the medium used the majority of H_2S negative enteric bacteria were producing H_2S. The degree of the reaction varied; in the "negative" group Klebsiella, Shigella, Providencia and Hafnia strains produced H_2S amounts detectable only by the sensitive lead acetate strips. Positive reactions were, however, definite in every group.

The results suggested that in media used for routine H_2S reaction, the substrate corresponded to inorganic sulphur compounds. The validity of this consideration was supported by the qualitative paper chromatography of autoclaved peptones. Of sulphur-containing amino acids only methionine was found in the peptone samples, the very substance which cannot be used by bacteria for H_2S production.

Experiments on media containing inorganic sulphur sources. In order to confirm the supposed role in H_2S production of inorganic sulphur compounds, first the peptones, the most probable sulphate-containing ingredients, were

tested. Quantitative analysis showed that any of the mentioned peptones contained relatively high amounts of sulphates.

Subsequently, it was examined whether inorganic sulphur compounds might be in reality substrated for H_2S production. The examinations were carried out on basal media prepared with peptones devoid of sulphates. Table II shows the results yielded by unsupplemented and inorganic sulphur-supplemented media.

Table II

Production of H_2S in agar medium prepared with sulphate-free and thiosulphate supplemented peptone. Indicator, 0.08% $(NH_4)_2 Fe(SO_4)_2$

Strains	No. of strains	H_2S production with			
		peptone sulphate-free		peptone supplemented with 0.04% $Na_2S_2O_3$	
		−	+	−	+
P. vulgaris	50	50	—	—	50
Salmonella	128	128	—	2	126
Arizona	1	1	—	—	1
Ballerup	1	1	—	—	1
Bethesda Na 1a ...	1	1	—	—	1
P. morganii	100	100	—	100	—
E. coli	139	139	—	139	—
Klebsiella	30	30	—	30	—
Shigella	72	72	—	72	—
Providencia	29	29	—	29	—

Table II contains only the reactions obtained with media prepared with sulphateless Richter peptone and with $Na_2S_2O_3$. It should be noted that experiments with other peptones, and with Na_2SO_4 or Na_2SO_3 as sulphur sources yielded similar results.

In view of the negative reactions obtained even with the lead acetate paper strip technique, it was clear that in the absence of inorganic sulphur also "H_2S positive" groups failed to produce H_2S. When the basal medium was supplemented with 0.04 per cent $Na_2S_2O_3$, H_2S production by "H_2S positive" strains was abundant. The same results were obtained with 0.04 per cent Na_2SO_4 or Na_2SO_3.

Medium for testing H_2S production. It was evident from the findings that, if grown in the presence of organic sulphur sources, every enteric bacterium is able to produce H_2S. In contrast, only a small part of these organisms, the H_2S producing genera, were able to reduce inorganic sulphur compounds. As peptones contain usable organic sulphur sources only in traces or not at

all, the result of the systematically very important test depends on the quantity of the peptone's sulphate contamination. The degree of impurity being variable, it is most important to use a standard medium. In our experience, optimal results are yielded by media prepared with sulphate-depleted peptone and standard concentrations of $Na_2S_2O_3$. The optimum concentration of $Na_2S_2O_3$ is 0.04 per cent. For preparing the medium, 1 per cent purified peptone (see "Materials and methods"), 0.5 per cent NaCl and 2 per cent washed agar are dissolved by heating. After filtration, as an indicator a 2 per cent solution of $(NH_4)_2Fe(SO_4)_2$ or $FeCl_2$ is added to give a final concentration of 0.08 per cent (4 ml in 100 ml medium). When tested in this medium the "H_2S positive" members of Enterobacteriaceae listed in the "Materials and methods" part of this paper, invariably gave definite positive reactions. Members of the "H_2S negative" groups never showed positive or doubtful results.

Discussion

Since the early days of bacteriology, H_2S formation has extensively been used for the identification of microorganisms. This important method was discovered by ORLOWSKI in 1895 [1]. It has been known for long that the sensitivity of the reaction varies with different peptone preparations. TILLEY in 1923 showed that the reaction did not depend on the cystine content of the medium and that an agar medium supplemented with thiosulphate was more suitable for diagnostic purposes [2]. In order to eliminate the differences due to various peptones, a number of media were empirically elaborated, many of which contained thiosulphate [3—5]. However, the proper indicator is not less important. The use of the most sensitive reagent, lead acetate, had been abandoned because of its toxicity. The advantages of bismuth and iron salts were stressed by HUNTER and CRECELIUS [6]. ZOBELL and FELTHAM applied lead acetate paper strips [7]. Others, as UNTERMOHLEN and GEORGI, recommended nickel and cobalt salts, and noted that different indicators and media yielded different results [8]. Iron salts, such as ferrous chloride [9], iron ammonium sulphate or citrate [10], are generally regarded as sensitive and reliable indicators. Some authors elaborated rapid methods for the H_2S test [11, 12].

The mechanism of H_2S formation has not yet been elucidated. From the literature and the present experiments the following conclusions can be drawn. The production of H_2S is due to the reduction of sulphur-containing amino acids [16] or inorganic compounds. Of the former substances mainly cysteine, but also cystine, are utilized. In contrast, methionine and, according to some authors, taurine, are unsuitable as substrates [17]. Of the inorganic sulphur compounds, sulphates and sulphites are uniformly usable.

Various bacteria have long been known to use the enzyme sulphhydrase for the production of H_2S from cysteine and related compounds [13, 14, 15]. As

shown in the present experiments, every enteric bacterium is capable of exerting this type of enzymic activity. In contrast, inorganic sulphur compounds are reduced only by a small number of organisms belonging to the "H_2S positive" genera.

On the basis of the above data and the results of our present experiments, the questions outlined in the introductory part of the present paper may be answered as follows. *P. morganii*, *P. rettgeri*, Providencia, Escherichia, Shigella and Klebsiella, which are regarded as H_2S negative enteric bacteria, contain cysteine sulphhydrase and are thus able to produce H_2S from cysteine and cystine. These bacteria, however, produce H_2S neither from thiosulphate nor from sulphate. In our and other authors' opinion the originally negligible cystine content of peptones decreases further during sterilization, and thus in the medium H_2S is produced at most in traces undetectable by the usual iron salt indicators. In contrast, "H_2S positive" organisms are able to produce H_2S both from amino acids (cystine, cysteine) and from inorganic sulphur compounds. Accordingly, for these bacteria the sulphate contamination of peptones constitutes the main sulphur source.

As to the mechanism of H_2S production, various problems (*e. g.* influence of pH and rH) still await elucidation. To elucidate the role and importance of these factors, further studies are needed.

As the H_2S reaction is an important differential diagnostic feature, it should be examined by methods giving positive results exclusively with organisms regarded on the basis of long experience as H_2S producers. This test constitues a basic criterion for the systematic classification of enteric bacteria. It is evident that the use of a medium containing sulphur sources utilizable by "H_2S negative" bacteria, would make one of our most important biochemical reactions worthless. Therefore, in accordance with EWING's opinion [20], the reaction can be performed properly only in media equivalent in sensitivity to those empirically elaborated for this purpose.

Recognizing the principle of the difference between "obligate" and "facultative" H_2S producers, the results have made it possible to develop a standard medium allowing the uniform evaluation of H_2S production. When preparing the standard medium, the peptones must be freed from the sulphate contamination and the medium supplied with a constant amount (0.04 per cent) of $Na_2S_2O_3$. As indicators of H_2S production, $(NH_4)Fe(SO_4)_2$ or $FeCl_2$ should be used. The medium prepared in this manner will yield uniform results.

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ALKALINE PHOSPHATASE ACTIVITY OF NORMAL AND POLIOVIRUS INFECTED HeLa CELLS

By

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Summary. The hydrolytic activity of the alkaline phosphatase of normal and poliovirus (Saukett strain) infected HeLa cells has been studied. Under standardized experimental conditions the phosphatase activity was found to be influenced by the age of the cultures.

In virus infected cells alkaline phosphatase activity varied with the individual phases of the viral cycle. Maximum activity occurred between the 3rd and 4th hours following infection The height of this maximum as referred to the corresponding base level was the same, indepen dently of the age of the culture. The virus yield, however, was the lower the older the culture. Thus it is supposed that alkaline phosphatase does not participate directly in poliovirus synthesis and that no specific alkaline phosphatase is synthesized on virus infection.

This statement was further supported by the similarity of Michaelis constants found for all alkaline phosphatases in the system. This seemed to exclude the possibility of formation of an inhibitor(s) during the eclipse phase of poliovirus replication.

The phosphorus metabolism of poliovirus infected tissue culture cells has been studied by a number of authors [1, 2, 3, 4]. Their results, particulary those yielded by enzymological examinations, are, however, contradictory, equal, increased or decreased activities having been found for the enzymes taking part in phosphorus metabolism (acid and alkaline phosphatase, and ribonuclease) in poliovirus infected cells as compared to normal ones [5, 6, 7, 8, 40].

Certain relations between age, enzyme activity, poliovirus sensitivity and virus yield of tissue culture cells have also been reported [5, 6, 9, 36]. These data, however, are also contradictory. On the other hand, the results of isotope studies of the phosphorus metabolism of poliovirus infected tissue cultures are unequivocal as to the increased intensity of phosphorus metabolism in the eclipse phase, and there being an intimate relation between virus yield, phosphorus incorporation and phospholipid synthesis [10, 11, 12, 13, 14, 15].

There are, however, certain contradictions between the results obtained by the enzymological and isotope methods.

The present study has been performed in order to establish the possible reasons for the contradictions in the literature. Examinations were made on the HeLa cell — poliovirus (Saukett strain) system in well defined phases of the virus replication cycle in cultures of given age, studying the virus yield as related to the hydrolytic activity of the alkaline phosphatase.

Materials and methods

Substrate. A 0.5 per cent (16.33×10^{-5} *M*) solution of sodium-b-glycerophosphate in *M*/15 veronal buffer pH 8.9 was used. The same buffer was used for preparing dilutions of the substrate.

Trypsin. For replicating or harvesting cells a 0.1 per cent solution of Difco (1 : 250) trypsin in Hanks' solution pH 7.3 was used.

Cells. The HeLa cell strain maintained through serial transfers in our laboratory was used throughout [24].

Virus. Strain Saukett of type 3 poliovirus maintained in HeLa cells was used.

Media. Medium No. I for routine transfers of HeLa cells consisted of 64 per cent Hanks' solution, 16 per cent human serum, 5 per cent lactalbumine hydrolysate from a 5% stock solution, 5 per cent Parker's medium 199 [22] and 3 to 5 per cent Gey C bicarbonate buffer.

Medium No. II for preparing monolayers contained 80 per cent Gey solution [23], 5 per cent lactalbumine hydrolysate from a 5 per cent stock solution, 5 per cent Gey C bicarbonate buffer and 5 per cent rabbit serum.

Medium No. III. added 1:1 v/v to a 2.7 per cent agar-agar solution, was composed of 20 ml 10× concentrated Gey solution, 40 ml 5 per cent lactalbumin hydrolysate, 20 ml rabbit serum inactivated at 56° C, 2 ml 20 per cent glucose, 15 ml Gey C bicarbonate buffer freshly saturated with CO_2.

Media I, II and III each contained 100 U of penicillin, 100 mg of streptomycin sulphate, and 25 U of nystatin per ml.

Virus assay. The number of plaque forming units (PFU) was determined in HeLa cell monolayers. Virus dilutions were prepared in Parker's 199 medium (pH 7.3) and appropriate dilutions in 0.1 ml were inoculated onto monolayers. After 30 minutes of adsorption at 37° C the cell layers were washed twice with 5 ml of diluent each and overlayered. After an incubation of 96 hours at 37° C the plaques were made visible by a 0.045 per cent indonitrotetrazolium chloride (Dajac) solution in a 1 ml agar overlay [25]. From each individual dilution, 5 plates were prepared in parallel.

Preparation of monolayers for enzyme assay. HeLa cell monolayers of appropriate age were infected by the Saukett strain of poliovirus at a multiplicity of 10. Adsorption was allowed to take place for 30 minutes at 37° C then the cells were washed twice with 5 ml of Parker 199 medium pH 7.3 and overlayered with 5 ml of the same medium. The plates were kept at 37° C for different periods of time. As controls, similarly treated uninfected cultures were used. At appropriate points of time the medium was replaced by trypsin and the incubation continued for 15 minutes. The cell suspension thus obtained served for the preparation of homogenates. Suspensions for one assay were prepared simultaneously from 10 monolayers.

Preparation of cell homogenates. The suspensions were centrifuged, washed in Hanks' solution, pH 7.3, and the cell count was determined in a Buerker chamber. The sediment was resuspended in distilled water so as to obtain 10^6 cells per ml. The cells were all lysed after a few pipettings. The suspension thus obtained was considered a cell homogenate.

Alkaline phosphatase assay. To one volume homogenate two volumes of sodium-b-glycerophosphate of adequate molarity and pH 8.9 were added after preheating in a water bath at 37° C. The enzyme-substrate mixtures were prepared in an amount of 60 ml and kept under constant stirring in a water bath of 37° C. Every 30 minutes 3 ml samples were taken and transferred immediately into 3 ml of a 10 per cent trichloroacetic acid solution.

The amount of anorganic phosphate produced by the enzyme was measured by FISKE and SUBBAROW's method [26]. Readings were taken at 60 μm in a Beckman spectrophotometer. For measuring enzyme activity, a substrate concentration of 27.21×10^{-4} *M* was used except when stated otherwise. The amount of substrate (in moles) split by a homogenate of 10^6 cells in 1 hour at pH 8.9 was taken as the unit of enzyme activity.

Results

In some preliminary tests the alkaline phosphatase activity of poliovirus infected HeLa cells exhibited a certain increase in the eclipse phase. Thus an attempt was made to establish the precise period during which the difference in alkaline phosphatase activity between equal numbers of normal and infected cells was maximal. This period has been found to be between the 3rd and 4th hours of the cycle (Fig. 1), when the synthesis of poliovirus is known to begin.

Thus one may reasonably suppose that at that point of time all the preparatory biochemical events have come to an end, while there is still no important loss in the normal protein content of the cells.

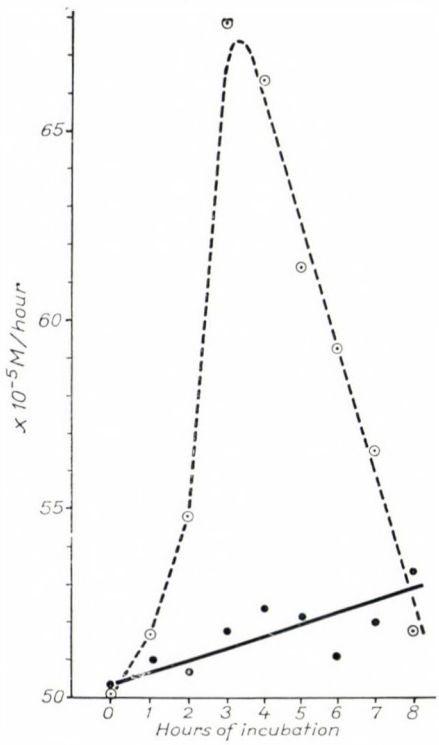

Fig. 1. Alkaline phosphatase activity in normal and infected HeLa cell cultures
——— normal; - - - - - infected

For these reasons, all enzyme preparations from infected cells were prepared at the end of the lag phase (3rd hour), except when stated otherwise.

Next, the influence of the age of cultures on the phosphatase activity referred to one cell was examined in 2, 4, 8 and 13 days old cultures. As demonstrated in Table I, enzyme activity was the higher the longer the cells had been cultivated [27, 28, 36]. The same was true for 2, 4, 8 and 13 days old cultures infected with poliovirus.

The difference in enzyme activity of normal and infected cultures as referred to an identical cell-count was always practically the same, independently of the age of the cells (see dV values of Table I). The virus yield of 2, 4 and 13 days old HeLa cells was determined in order to elucidate any possible relation between virus producing capacity and alkaline phosphatase activity. The results are given in Table II.

Table I

$V\left(\frac{dc}{dt}\right)$ and dV values for alkaline phosphatase of normal and infected HeLa cell cultures of different ages

Age of HeLa cell cultures, days	$V = \frac{dc}{dt}$ ($\times 10^{-5}$ M/hour)		dV
	Normal	Infected	
2	2.675	4.162	1.487
4	4.312	5.810	1.498
8	3.044	4.749	1.705
13	5.677	6.993	1.316

Table II

Poliovirus yield of HeLa cell cultures of different ages. Plaque forming units per cell

Age of HeLa cell cultures, days	PFU/cell
2	188 ± 3
4	125 ± 5
13	92 ± 4

The data of Table II show that the virus yield of a 13-day old culture was 50 per cent less than that of a 2-day old one. The decrease of the capacity to support virus replication and a lowering of sensitivity to poliovirus with the ageing of cells of HeLa cultures has been observed also by other authors [27, 28].

No correlation was found between the virus producing capacity and the alkaline phosphatase activity of cells. Thus it seems improbable that the alkaline phosphatase would directly participate in the synthesis of poliovirus or that a specific "viral" alkaline phosphatase should be produced. In order to exclude such a possibility, the Michaelis constants (K_m) of the alkaline phosphatases found in normal and infected HeLa cells were determined by the graphic method of LINEWEAVER and BURK [29].

In addition, the statement of some authors [5, 6, 7, 40] about the appearance of an alkaline phosphatase inhibitor in the infected cultures was reexamined.

When determining the K_m values the age of the cultures was taken into account. Results obtained for 2, 4, 8 and 13 days old normal and infected cells are given in Table III. The maximum values of V are presented in Table IV.

As revealed by the data of Table III, a certain change in the K_m value of alkaline phosphatase of normal cells occurs between the 2nd and 4th days of

cultivation. From the 4th day on, however, the K_m value remains unchanged up to the 13th day, the latest point of time examined. It should be noted that virus infected cultures behave similarly and only 2 day old cultures exhibit a different K_m value. The enzymes from older infected cultures do not differ in K_m either from each other or from the normal enzyme.

We found no inhibitor that would have influenced the hydrolytic activity of alkaline phosphatase from infected cultures.

The K_m values have shown that the increased enzyme activity of infected cultures is not due to the appearance of a new enzyme but to the production of increased quantities of the normally present alkaline phosphatase.

Table III

Michaelis constant values for alkaline phosphatase of normal and infected HeLa cell cultures of different ages

Age of HeLa cell cultures, days	Michaelis constant ($\times 10^{-5}$ M)	
	Normal	Infected
2	10.43	45.92
4	21.96	22.00
8	18.57	22.43
13	23.64	25.97

Table IV

V max. and $d\,V$ max. values for alkaline phosphatase of normal and infected HeLa cell culturesof different ages

Age of HeLa cell cultures, days	V max. ($\times 10^{-5}$ M/hour)		dV max.
	Normal	Infected	
2	2.820	4.834	2.014
4	4.770	6.472	1.702
8	3.335	5.215	1.880
13	6.217	8.430	2.213

Discussion

In agreement with data in the literature [5, 9, 36], the hydrolytic activity of the alkaline phosphatase of HeLa cells, as referred to a definite cell count, has been found to be correlated with the age of the cultures and the activity of the enzyme. In poliovirus infected cultures alkaline phosphatase activity was found to have increased. The extent of this increase depended on the actual phase of the viral cycle; the peak fell between the 3rd and 4th hours after

infection. This observation is in agreement with those of MIROFF [10], CORNATZER [11, 12, 13], HILTON [14] and TOHÁ [15], who found maximum P^{32} incorporation, maximum phospholipid synthesis and RNA turnover in the period between 3.5 and 4 hours of the viral cycle.

Certain authors have already pointed to the fact that alkaline phosphatase catalyses not only the intracellular hydrolysis of phosphate esters but also participates in the incorporation of anorganic phosphate [16, 17] and in certain phosphorous transfer reactions [18, 19, 20, 21, 37, 41]. Thus the increased alkaline phosphatase activity found in poliovirus infected HeLa cells may be regarded as a sign of increased phosphorus metabolism resulting from enhanced membrane transport [11], extensive structural reorganisation [38, 39, 42] increased oxidative phosphorylation [11, 13], or enhanced phosphorus transfer [16—21, 37].

The difference in enzyme activity of normal and poliovirus infected cells was remarkably constant, in spite of the great fluctuations connected with the age of the cultures. The virus yield was considerably influenced by the age of the cells, although the lack of correlation between alkaline phosphatase activity and virus yield favours the suggestion that the enzyme does not participate directly in virus synthesis. Increased alkaline phosphatase activity should thus be regarded as a secondary phenomenon.

On the other hand, ZASLAVSKY [9] found a correlation between age, alkaline phosphatase activity, and poliovirus sensitivity of the cultures. Our observations differ also from those of BACHTOLD [8], KOVÁCS [5, 6, 7], and DEIG [40]. BACHTOLD [8] did not find a change in enzyme activity in poliovirus infected monkey kidney cells until their degeneration when, however, activity decreased abruptly. KOVÁCS [5, 6, 7], and DEIG [40] observed a decreased alkaline phosphatase activity in monkey kidney cells infected with poliovirus. These authors supposed the appearance of an inhibitor. These observations, however, were not made in the eclipse phase of the cycle, nor was the inoculum as high as in our experiments. The latter factor might have resulted in a non-one-step type of growth curve. Thus we think that the asynchronism of biological and biochemical events, together with the considerable protein loss occurring later in the viral cycle, may render results obtained under such conditions questionable.

Under our experimental conditions the K_m values for the alkaline phosphatase of poliovirus infected cells did not reveal the presence of any inhibitor. This observation is in contrast with that of KOVÁCS [5, 6, 7].

The practically identical K_m values for the alkaline phosphatase of infected and control cells of any age suggest the lack of formation of any "new" type of that enzyme in poliovirus infected HeLa cells.

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CANDIDA SOOSII N. SP., A NEW YEAST RELATED TO CANDIDA REQUINYII SZÉP ET NOVÁK 1963

By

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Summary. A new yeast isolated as an industrial fermentation contaminant has been described. The new species, *Candida soosii*, named in honour of *Professor* I. Soós, belongs to the *Candida requinyii* group of genus Candida. *C. soosii* produces no ascus, reproduces with budding and pseudomycelium (forms no mycelium and arthrospore); does not produce starch and carotenoid pigments; ferments glucose and galactose (the latter weakly and slowly); utilizes glucose, galactose, sucrose, and ethanol as a sole source of carbon and does not decompose arbutin.

In examining the ecology of yeasts, a "wild" strain isolated from an industrial fermentor has been identified as different from known species, but related to the recently described *Candida requinyii* Szép et Novák 1963 [9]. The present paper describes this organism and its relationship to *C. requinyii*.

Materials and methods

The strain has been isolated and maintained by *Dr.* F. Simek (Industrial Food Research Institute, Budapest) on malt agar of 10° Balling. In order to obtain a pure culture, the strain was streaked onto Pagano, Levin and Trejo's TTC agar [7]. Identification was performed by the methods of Lodder and Kreger van Rij [2] modified by Zsolt and Novák [10]. Taxonomic classification was made on the basis of Novák and Zsolt's system [5].

Alkalization in peptone water was tested by Serény's method [8], in 1 ml amounts of pH 4.1 and pH 4.7 media containing 1 per cent Bacto peptone (Difco), 0.5 per cent NaCl and 0.0024 per cent bromcresol purple (to 1000 ml medium 12 ml indicator solution containing 0.2 g BCP and 5 ml *N* NaOH made up to 100 ml was added). The tubes were incubated at 26° C under aerobic conditions.

Results and discussion

(i) *Taxonomically important properties:* (1) Ascospore formation: nil; (2) Vegetative reproduction: by budding and pseudomycelium formation. Mycelium and arthrospores are not produced; (3) Carbohydrate fermentation: d (g)/smlr (Numerator: positive reactions, Denominator: negative reactions. d, D = glucose, g, G = galactose, s, S = sucrose, m, M = maltose, l, L = lactose, r, R = raffinose. Small letters refer to fermentation, capital letters to assimilation. Bracketed letters indicate weak or latent reactions.); (4) Carbohydrate assimilation: DGS /MLR; (5) Ethanol assimilation: + (pellicle formation); (6) Nitrate

assimilation: —; (7) Arbutin decomposition: —; (8) Starch production: —; (9) Carotenoid pigment production: —.

(ii) *Other properties.* (1) Growth in malt extract, 26° C, 3 days: cells are oval or long oval, 2.33—4.66 by 4.66—9.33 μ singly or in pairs (small cells). A thin, somewhat rugose creeping pellicle and a sediment are formed.

Table I

The taxonomic position of Candida soossi n. sp. in the Candida requinyii species-group [6]

Species	Assimilation						Fermentation						Assimilation		
	Glucose	Galactose	Sucrose	Maltose	Lactose	Raffinose	Glucose	Galactose	Sucrose	Maltose	Lactose	Raffinose	Nitrate	Ethanol	Arbutin
C. requinyii SZÉP et NOVÁK	+	+	+	—	—	—	+	—	—	—	—	—	—	+	+
C. soossi n. sp.	+	+	+	—	—	—	+	(+)	—	—	—	—	—	+	+
C. trigonopsoides DIETRICHSON ..	+	+	?	—	—	—	+	—	?	—	—	—	—	+	+

? Contradictory data in original publication (see NOVÁK and ZSOLT [6], and SZÉP and NOVÁK [9]).

17° C, 30 days: A thick, somewhat creeping pellicle sinking down with age, is formed.

(2) Growth on malt agar, 26° C, 3 days: the colony is dull, dry, light cream-coloured, the edge is finely crenated. Cells are oval, long oval or cylindrical (some of them are filamentous) singly or in pairs, forming sometimes short branching chains; 2.30—4.66 by 3.50—18.33—46.66 μ.

17° C, 30 days: The colony is cream-coloured dull, somewhat raised in the centre, with granulated surface, the edge fringed by pseudomycelium.

(3) Potato-glucose agar slide cultures: Budding cells and pseudomycelium are seen.

(4) Corn-meal agar slants [4]: Budding cells and pseudomycelium, but no true mycelium, arhtrospores or chlamydospores can be observed.

(5) Growth on PAGANO—LEVIN—TREJO TTC agar: The colonies are white, dry, dull and the edges are somewhat fringed by pseudomycelium.

(6) Growth in peptone water:

pH 4.7: Alkalization 1 day, strong alkaline reaction in 3 days.

pH 4.1: Very weak alkalization 3 days. Medium reaction in 1 week.

Considering the absence of ascospore, true mycelium, arthrospore, carotinoid pigment, starch and intensive acid production and the presence of budding, pseudomycelium formation and fermentation, the examined strain was classified in the genus Candida Berkhout emend. NOVÁK and ZSOLT [5]. The organism, however, differs in biochemical reactions from the known species of

the genus. Considering its other properties, it stands very close to *C. requinyii* Szép et Novák [9]. The new species differs from the latter with its latent and weak galactose fermentation. Thus, according to Novák and Zsolt's new classification [6], it may be included in the species-group *Candida requinyii* (Table I). As to species groups, the possibility of synonymization of species has been considered and in certain cases the differences in fermentation reactions have not been accepted as criteria for differentiation [6], therefore in the present study alkalization in peptone water, which definitely differed with

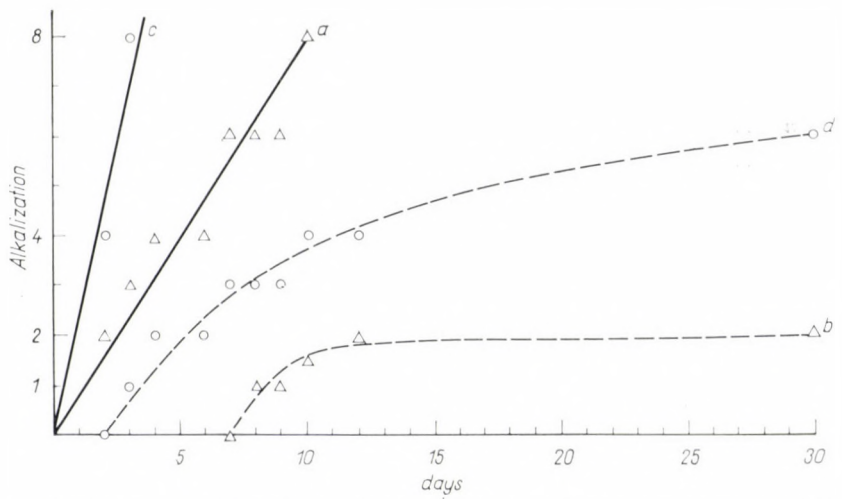

Fig. 1. Alkalization in pH 4.7 and pH 4.1 peptone water by *Candida requinyii* Szép et Novák and *Candida soosii n. sp.*
a = *C. requinyii* pH 4.7; *b* = *C. requinyii* pH 4.1; *c* = *C. soosii* pH 4.7; *d* = *C. soosii* pH 4.1
8 = strong; 4 = definite; 2 = weak; 1 = very weak

C. soosii and *C. requinyii* (Fig. 1), was also considered. The difference between the two species is indicated further by the immunofluorescence studies of Novák and Geck [3], in which *C. requinyii* gave a +++, *C. soosii* a ++ fluorescence with undiluted fluorochromized anti-albicans rabbit serum.

It should be noted that up to now only one yeast species with a behaviour similar to that of *C. soosii* has been known. This organism, *Endomycopsis dermatensis* Batista, Campos et Coelho [1], without alteration of the original description, should be classified according to our system as *Prosaccharomyces dermatensis* (Batista, Campos et Coelho) nov. comb. From Table II it is evident that in addition to ascospore formation, there is a difference between the two organisms in the formation of true mycelium.

On the basis of the presented data, the examined strain should be included as a new species in the species-group *Candida requinyii* of the genus Candida (Berkhout) Novák et Zsolt [5]. The recommended name for the

organism is *Candida soosii* in honour of *Professor I.* Soós, in remembrance of his successful contributions to Hungarian oenology and yeast research.

Table II

Main systematic properties of Prosaccharomyces dermatensis (BATISTA, CAMPOS et COELHO) *nov. comb. and Candida soosii n. sp.*

	Production of asci	Shape of ascospores	Surface of ascospores	Number of ascospores	True mycelium	Arthrospore	Budding	Pseudomycelium	Assimilation						Fermentation						Assimilation		
									Glucose	Galactose	Sucrose	Maltose	Lactose	Raffinose	Glucose	Galactose	Sucrose	Maltose	Lactose	Raffinose	Nitrate	Ethanol	Arbutin
P. dermatensis . .	*h*	*g*	/	1–8	+	–	+	+	+	+	+	–	–	–	+	+	–	–	–	–	–	+	/
C. sossii n. sp. . .	–	–	–	–	–	–	+	+	+	+	+	–	–	–	+	(+)	–	–	–	–	–	+	–

Latin diagnosis. Asci et ascosporae non formantur, gemmae et pseudomycelium sed non mycelium verum arthrosporaeque formantur.

Glucosum et galactosum (exiguum et leniterum) fermentatur. In medio minerali cum glucoso, galactoso, saccharo et alcohole ethylico crescit. Nitras kalicus non assimilantur. Amylum et pigmenta carotinoidea non componitur. Arbutinum non finditur. In aqua peptonato apud pH 4.7 bene crescit, apud pH 4.1 exigue crescit.

In musto maltato (26° C post tertiem diem) cellulae gemmantes ovoideae vel longovoideae (2.33—4.66×4.66—9.33 μ) singulae aut binae. Sedimentum pelliculaeque formantur. In agaro maltato post tertiem diem (26° C) cultura flavalbida, surda, non nitida, margine piloso; post unum mensem (17° C) cultura flavalbida, surda, acclivis, margine piloso.

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PRODUCTION OF A LYTIC FACTOR BY ULTRAVIOLET LIGHT IRRADIATED CULTURES OF BACILLUS CEREUS

II. SOME PROPERTIES AND QUANTITATIVE DETERMINATION OF THE LYTIC FACTOR

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Summary. A quantitative method for the determination of the lytic factor produced by ultraviolet irradiated *B. cereus* strain 569 has been elaborated. The mechanism of the lytic action of this factor on *B. cereus* 130 has also been investigated.

Studies on the properties of the lytic factor indicated its enzymic nature. The adsorption of the agent on *B. cereus* 130 cells has been found essential for the accomplishment of lysis.

In a previous paper the lytic activity of ultraviolet irradiated *B. cereus* strain 569 has been reported. The lysate attacked only vegetative cells of *B. cereus* strain 130, while it was unable to lyse the vegetative form of strain 569 [1].

Bacterial enzymes lysing the bacterial cell wall are frequently encountered. Some of them are released from sporulating cultures, others are formed within the host cell — bacteriophage system. The latter agents are liberated only after the lysis of the cell.

In view of its proteinic nature and its capability of producing the lysis of *B cereus* 130, our lytic factor may be supposed to belong to one of the above types of bacterial enzymes. In order to support this view, a method suitable for analysis and quantitative determination of the phenomenon has been needed. The present paper gives an account of a new quantitive method, which has made it possible to recognize some new properties of the lytic factor.

Materials and methods

Measurement of lytic activity on B. cereus 130. A *B. cereus* 130 suspension giving an optical density reading of 0.5, was centrifuged, washed in one volume of saline, then resuspended in one half volume of 0.16 *M* NaCl. The lytic system contained in a final volume of 10 ml: 4 ml cell suspension, 4 ml 1 per cent gelatin, 1 ml pH 8, 0.1 *M* veronal Na—HCl buffer, 0 to 1 ml lysate and, if required, medium to complete it to 10 ml.

Gelatin served to decrease precipitate production during the lysis of the cells and the action of proteolytic enzymes on the lytic factor. The degree of lysis was measured at various intervals by reading the optical density in the Unicam spectrophotometer at 620 mμ and room temperature in 1 cm cuvettes. The mg/ml dry weight content was obtained by multiplying by 0.25 the read optical density values.

Trypsin resistant preparation (TRP) was produced by use of methods generally applied for cell wall preparation. Disintegration of the cells was performed with a sonic oscillator, then protein and rbonucleic acid digestion and washing were carried out as described by McCarty [2]. *B. cereus* 130 was grown until an optical density value of 2—2.5 was reached (0.5—0.6 mg/ml dry weight); then the cells were centrifuged and resuspended in one tenth volume of 1

per cent NaCl. Sonic treatment of the concentrated suspension lasted for 5 minutes at 0° C. After centrifugation at 2000—3000 *g* for 5 minutes, the supernatant was pipetted out, and, in order to obtain the cellular debris present in this fraction, it was centrifuged again at 10 000 *g* for 10 minutes. The deposit was suspended in the above volume of 1 per cent NaCl and subjected to a second centrifugation at 10 000 *g* for 10 minutes. The deposit was incubated in 0.1 *M*, pH 8 phosphate buffer in the presence of 1 mg/ml trypsin. The degree of digestion was estimated from the decrease in density caused partly by denatured proteins. The system was incubated for about 1 to 2 hours until the optical density decreased. Then trypsin resistant substances were separated from the supernatant by centrifugation at 10 000 *g* for 10 minutes. The deposit was suspended in 0.1 *M*, pH 8 phosphate buffer. Further purification was carried out with 0.5 mg/ml ribonuclease at room temperature. Samples were taken at half-hour intervals, centrifuged and the optical density of the supernatant was measured photometrically at 260 mμ. Ribonuclease treatment was continued until the optical density value had become constant. TRP was then separated by centrifugation at 10 000 *g* for 10 minutes. The deposit was a water-insoluble, white substance. Electronmicroscopically, it consisted of cell wall particles. For storing, the preparation was suspended in 0.1 *M*, pH 8 phosphate buffer.

Measurement of lytic activity on TRP. The measurements were carried out similarly to those described for *B. cereus* 130. The reaction mixture contained in 5 ml final volume 2 ml TRP (optical density, 1.25), 2.5 ml of 1 per cent gelatin, 0 to 0.5 ml of lysate and, if required, it was completed to 5 ml by the medium.

The degree of lysis was estimated by use of the Unicam spectrophotometer at 620 mμ.

In the course of TRP-lysis, the optical density of the system decreased by 80 to 90 per cent. The degree of decrease served at the same time for estimating the purity of TRP, as lower values — at maximum lysis — were considered to be caused by contaminating substances insensitive to the lytic factor. The optical density values were calibrated for dry TRP content.

Measurement of lytic activity by serial sampling. When continuous measurement of the lysis could not be performed, optical density values were determined in samples taken at given intervals. The lytic action was stopped by acidifying the system to below pH 4.5. At acid reaction the optical density remained unaltered for 1 to 1½ hours, thus within this period the samples could be collected for serial measurement.

Heating of B. cereus 130. Suspensions giving optical density readings of 0.5—1, were centrifuged then the deposit was suspended in 1 per cent NaCl and heated for 10 minutes in 100° C water bath.

Strains and cultivation methods were as described previously [1].

Experimental

Action of the lytic factor on B. cereus 130. The lysis of sensitive bacteria is widely used for the detection and quantitative determination of various lytic enzymes. Thus different bacteria are used for estimating the lysozyme [3], *E. coli* endolysin [4], and the enzyme released from sporulating cells [5, 6]. Our method was principally identical with these techniques. The lysis of the indicator strain, *B. cereus* 130, in the presence of various amounts of the lytic factor was investigated. As shown in Fig. 1, the higher the concentration of the lysate, the more rapid was the lysis of the culture. It is seen that the rate of lysis was proportional to the amount of lysate. The initial part of the curve was linear, with slopes of 0.66 and 1.4 for 0.1 and 0.2 ml lysate, respectively. With higher amounts of lysate, however, the slope did not exhibit a linear relationship to the amount of lysate. Detection of the lytic substance with this methods could be accomplished only within one experiment, as in repeated experiments the same amount of lysate caused either quicker or slower lysis of strain 130. This finding may be explained by the fact that the lysis of even the same indicator strain depends on several different factors.

In determining the lytic factor with strain 130, the role of an autolytic system should also be considered, as under our experimental conditions the test strain showed a tendency for spontaneous lysis. The lytic effect also varies with the predomination of catabolic or anabolic processes. The lysis of strain 130 growing actively at 37° C could be produced only with undiluted or at most 1 : 10 diluted lysate. In contrast, resting cells were effectively lysed even by 1 : 200 dilutions. Dinitrophenol increased the rate of lysis. The lytic activ-

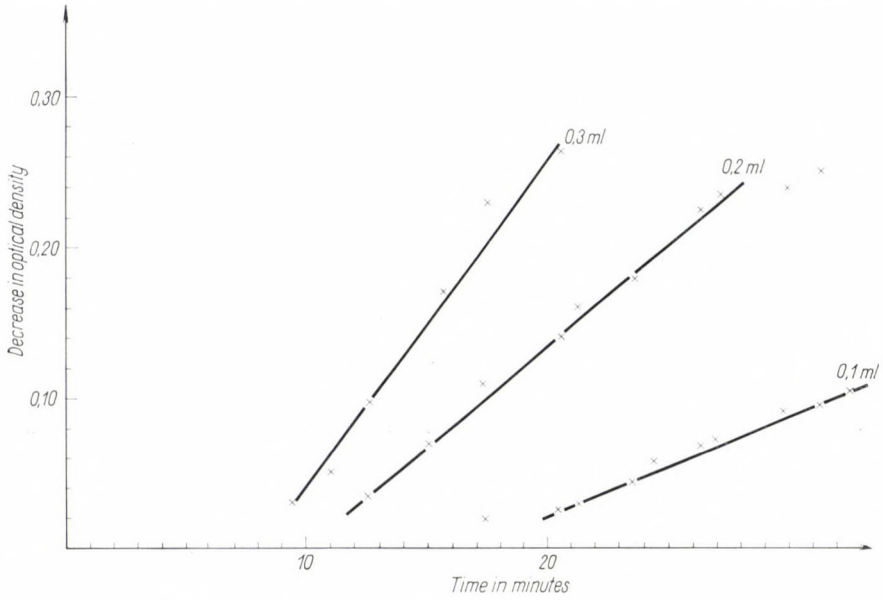

Fig. 1. Effect of different amounts of lysate on B cereus 130 cells

ity was independent of protein synthesis, as it was not influenced by 5—100 μg/ml concentrations of chloramphenicol.

Mechanism of the lysis of B. cereus 130. As shown by these findings, the lytic action exerted by our preparation is a complex process. Bacterial lysis is known to be due to an activation of the cell's own autolytic system [7]. It has been questionable whether in the case of strain 130, in the reaction of which autolysis is undoubtedly concerned, an activation of the autolytic system or a direct decomposing effect of the lysate occurred. Heating experiments provided a definite elucidation of this problem. In studying the lysis of Gram positive cells, SALTON showed that boiled cultures were rapidly lysed by trypsin only after lysozyme treatment [8].

Fig. 2 shows that the cells of boiled strain 130 were not attacked by trypsin. The degree of lysis was relatively low when the lytic factor acted upon the heated cells. When trypsin was added after lytic factor treatment, the lysis

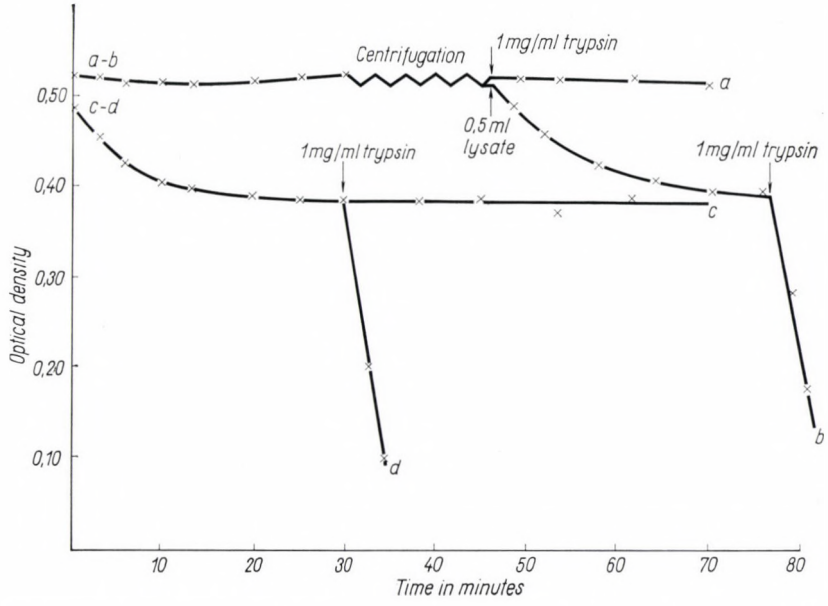

Fig. 2. Effect of trypsin on boiled *B. cereus* 130. Curve *a—b:* Cells were treated with 1 mg/ml trypsin, centrifuged after 30 minutes, then resuspended and divided into two parts; part (*a*) was treated with 1 mg/ml trypsin, part (*b*) with 0.5 ml lysate. Curve *c—d:* Cells were treated with 0.5 ml lysate. When the decrease in optical density had ceased, the system was divided into two parts; to part (*d*) 1 mg/ml trypsin was added

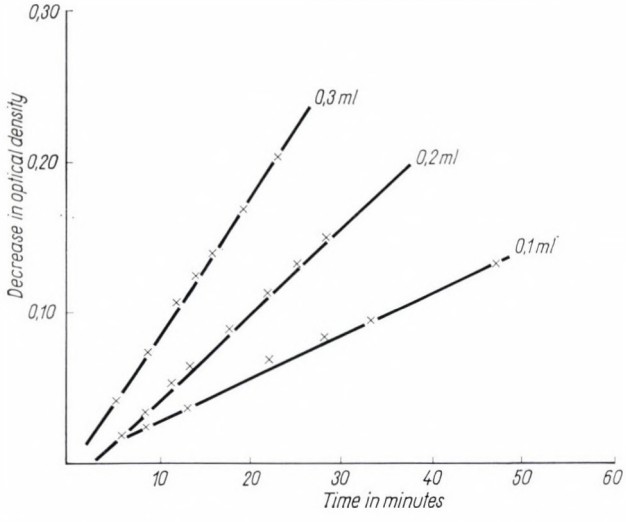

Fig. 3. Effect of different amounts of lysate on TRP. Slope for 0.1 ml lysate, 0.3; for 0.2 ml lysate, 0.57; for 0.3 ml lysate, 0.88

was rapid and almost complete. The alternative of this, however, failed to promote the lysis, as after tryptic digestion and subsequent removal for the enzyme, the lytic factor caused no stronger reaction than that obtained without trypsin pre-treatment. Thus it is clear that trypsin does not act upon the substrate of the lytic factor, and *vice versa*. On the other hand, the lytic effect of our substance appears only when its action is completed by a proteolytic agent, which *in se* is also incapable of exerting a lytic action. On comparing this

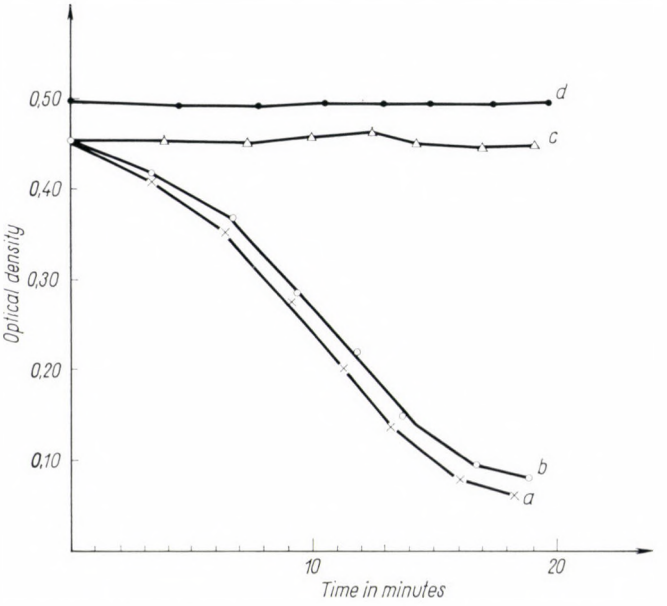

Fig. 4. Adsorption of the lytic factor by TRP. After centrifugation of 4.5 ml TRP (optica-density, 0.5), to the deposit 0.5 ml lysate and 4.5 ml 2 *M* NaCl were added at —3° C. To susl pend the lytic effect, centrifugation for 10 minutes was carried out at a similar temperature. The supernatant was separated and used as „supernatant 1", then the deposit was resuspended in 5 ml of 1 per cent NaCl, left to dissolve at room temperature and used as "supernatant 2". Lytic activity was tested on *B. cereus* 130. Curve *a:* activity of 0.1 ml lysate. Curve *b:* Activity of 1 ml supernatant 2. Curve *c:* Activity of 1 ml supernatant 1. Curve *d:* Untreated *B. cereus* 130

experiment with that of SALTON [8] for the lysis of lysozyme-treated *M. lysodeicticus* and *B. megatherium*, it is seen that our lytic factor and lysozyme act very similarly. Therefore it may be assumed that the lytic factor is also a cell wall-decomposing enzyme, and it may be expected that usual methods of cell wall preparation yield a fraction lysable by this agent.

Quantitative determination of the lytic factor by means of TRP. The measurement was carried out similarly to the method of STRANGE and DARK [9] described for estimation of the activity of enzymes produced in sporulating *B. cereus* cultures. This technique involves the determination of the lytic activity of enzymes on *B. cereus* cell wall preparations. Preliminary experi-

ments have shown that the rate of lysis is in direct proportion to the amount of the lytic factor (Fig. 3). As with higher concentrations of the agent no such correlation was found, an empirical curve was drawn. For this purpose the slopes obtained with different concentrations of lysate were plotted against the amounts of the lytic factor.

The fact that TRP is dissolved by the lytic factor does not necessarily mean that the mechanism of the reaction is identical with lysis of *B. cereus* 130. The identity of the agents acting upon strain 130 and TRP is indicated by the finding that the activity of the lysate is stopped against both, when the medium is adjusted to a pH lower than 4.5. The fact that TRP binds the lytic factor and after centrifugation the supernatant is incapable of acting on *B. cereus*, definitely confirms this consideration (Fig. 4, curve c). However, resuspension, the precipitated TRP is dissolved and an unaltered activity is found in the supernatant (Fig. 4, curve b). This experiment shows that the lytic substance is adsorbed on TRP, and the absorbed agent is effective on TRP itself. Its appearance in the supernatant indicates that the agent is not used up in the reaction.

The TRP method for the determination of lytic activity presents the advantage of reproducibility. Under identical experimental conditions, samples of the same batch of TRP are always lysed at identical rates and thus the results of various serial assays are comparable. For obtaining reproducible results with different TRP batches, preparations of identical optical density, dry substance content and rate of lysis would be necessary. As different preparations generally do not meet these requirements, for every batch a new calibration curve should be prepared.

Some properties of the lytic factor. The quantitative method allowed the elucidation of some characters of the lytic reaction. The lytic activity as a function of pH is presented in Fig. 5. The highest effect was exerted between pH 7.5 and 8.5. The lytic activity was expressed at the corresponding pH value as the slope obtained from the rate of lysis in the presence of equal amounts of lysate. The optical density of TRP suspensions at different pH values was approximately identical. This was checked also after the partial lysis, by acidifying or alkalizing the reaction mixtures by 2—3 pH degrees.

The influence of temperature on lytic activity was measured with TRP and *B. cereus* 130. Both measurements revealed the maximum activity between 20 and 40° C. Within this range no important activity changes were found. At 0° C the activity was nil, while at 10° C it was considerable. A definite decrease occurred at higher temperatures.

In assays carried out with TRP the relationship between lysis and substrate concentration could be examined only within a limited concentration range. As the reaction was performed in a heterogeneous system, TRP, could not be added beyond a certain limit. Measurements were carried out between 0.69

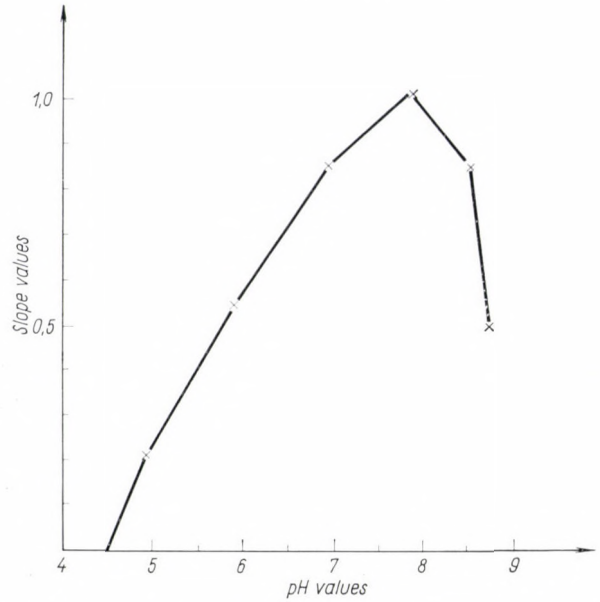

Fig. 5. Lytic activity of equal amounts of lysate on TRP at different pH values. Veronal Na—Na acetate buffer, 1/7 *M*

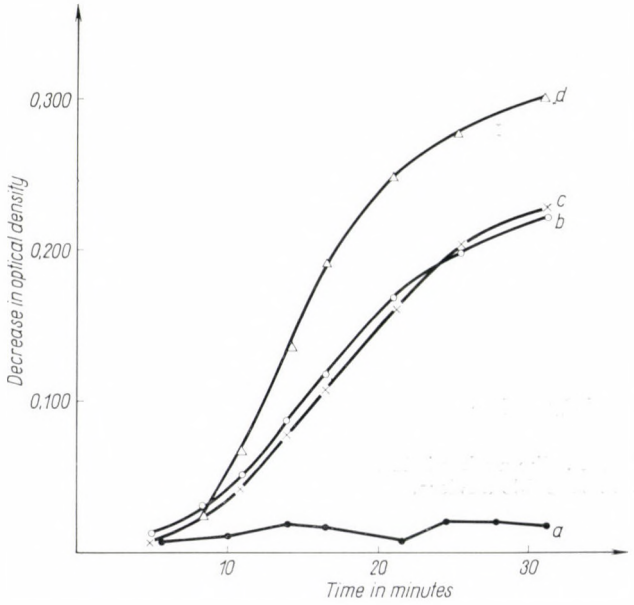

Fig. 6. Adsorption of the lytic factor on *B. cereus* strains.
Curve *a:* Activity of supernatant after adsorption on *B. cereus* 130; Curve *b:* Activity of supernatant after adsorption on *B. cereus* 114; Curve *c:* Activity of supernatant after adsorption on *B. cereus* 569; Curve *d:* Activity of 0.3 ml lysate on *B. cereus* 130

and 5.6 mg/ml concentration. It was found that, by increasing the TRP concentration at unaltered lysate concentrations, the amount of decomposed TRP could be increased without reaching saturation.

Adsorption of the lytic factor. The question arose a specific correlation existed between the cell and the active material. It has been shown that the lytic factor is adsorbed on *B. cereus* 130 and thus by centrifugation it can be removed from the solution. Quantitative conditions are presented in Fig. 6. When the lysate is applied in amounts causing no immediate lysis, at centrifugation a considerable part of the lytic factor will sediment with the cells. As shown by Fig. 6, *B. cereus* strains 569 and 114, which were less sensitive to the lytic action, adsorbed the agent slightly or not at all, and therefore the activity of the supernatant remained almost unchanged. After the lysis of strain 130, the lytic factor was liberated and, indicating that it was not used up in the reaction, was present in an active form. These experiments have shown that adsorption on cells and lytic activity are closely associated.

Discussion

The experiments have shown that the lytic acitivity exerted by our agent starts with an enzymic effect on the cell wall. MAXTED [10] showed in cultures infected with streptococcal C phage a substance capable of lysing boiled streptococci. The enzymes of sporulating *B. cereus* studied by STRANGE and DARK [9] attack the cell wall but have a limited effect on vegetative cells. MURPHY [11] in phage-infected *B. megatherium* cultures found a substance which lysed the cell walls. WEIDEL and PRIMOSIGH [12] studied the lytic enzyme of T phage acting on the cell wall of *E. coli*. ADAMS and PARK [13] showed in the lysate of *Klebsiella pneumoniae* a substance acting also on the cell wall. Our lytic factor is similar in its mode of action to those agents, being active also on the cell wall. The similarity is proved by the enzymic character of the lytic factor, too, as shown by kinetic measurements. The fact that the lytic factor is liberated during ultraviolet irradiation-induced lysis, and the specific nature of its adsorption to the sensitive cells, shows it to be a substance similar to the lytic enzymes of bacteriophages.

Acknowledgement. The author is indebted to *Professor* F. B. STRAUB and *Dr.* G. DÉNES for their interest and valuable advices.

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KONGRESS DER UNGARISCHEN MIKROBIOLOGISCHEN GESELLSCHAFT
28.—30. Oktober 1963

ZUSAMMENFASSUNGEN

ALLGEMEINE MIKROBIOLOGIE

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(Mikrobiologisches Institut der Medizinischen Universität Pécs)

KÜNSTLICHE SHIGELLA INFEKTION BEI MÄUSEN

Nach Elimination der Coli-Flora mit Streptomycin gelang die Ansiedlung von streptomycinresistenten Shigella-Stämmen *(Sh. flexneri* und *Sh. sonnei)* bei Mäusen. Nach der peroralen Shigella-Infektion wurden die unter sterilen Kautelen gehaltenen Mäuse weiterhin mit Streptomycin behandelt. Die auf diese Weise behandelten Mäuse waren — selbst Monate hindurch — symptomfreie permanente Bakterienausscheider. Im Darmtrakt der permanent ausscheidenden Mäuse waren pathomorphologische Veränderungen nicht anzutreffen. Nach 2—3wöchiger Ausscheidung konnten inkomplette Serum- und Copro-Agglutinine sowie protektive Antikörper demonstriert werden. Die Immunerscheinungen sprechen gegen den nicht ganz passiven Charakter des Prozesses. Bekräftigt wird diese Auffassung durch die Tatsache, daß es auch gelang, eine Mischinfektion zwischen *Shigella flexneri*-Typen (2*a* und 3) sowie *Sh. flexneri* und *Sh. sonnei* zustande zu bringen und weiterhin die Antikörperproduktion gegen beide Agenzien nachzuweisen. Das Erscheinen von *E. coli* oder *Klebsiella* im Darmtrakt der Mäuse führt zum Aufhören der Shigella-Ausscheidung, woraus die Schutzrolle der normalen Flora hervorgeht.

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DIE VARIATION DER ANTIGENSTRUKTUR VON SH. FLEXNERI BEI PERMANENT AUSSCHEIDENDEN MÄUSEN

Bei den per os mit *Sh. flexneri* 2*a* und 3-Stämmen infizierten Mäusen sind von der 3—4. Woche der Ausscheidung an Veränderungen in der Antigenstruktur zu beobachten. Die antigenstrukturellen Veränderungen in vivo folgen größtenteils den in vitro bekannten antigenstrukturellen Veränderungen. Bei *Sh. flexneri 3* verläuft die Endform der Variation in der Mehrzahl der Fälle in Richtung »X«, und häufig ist zu Beginn der Veränderung der Verlust des typusspezifischen Antigens (H) wahrnehmbar. Eine andersartige Veränderung mit Verlust der Komponenten »H« und »VII« ist auch in Richtung des Gruppen-

antigens (III) zu beobachten. Bei *Sh. flexneri 2a* ist das Endprodukt der Variation im allgemeinen die »Y«-Variante. In vielen Fällen läßt sich die Degradation graduell durch Verlust der einzelnen VIII-Komponenten verfolgen. Mitunter war der Verlust der gruppenspezifischen Antigene unter Bewahrung des typusspezifischen Antigens (D) zu beobachten.

Die antigenstrukturelle Veränderung kann im wesentlichen als eine Degradation in R-Richtung aufgefaßt werden. Wahrscheinlich bildet das Erscheinen der Antikörper den für den Abbau der Antigenkomponenten verantwortlichen Faktor.

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ÜBER DIE ANTIGENSTRUKTUR DER ZELLEN IN DER II. SH. SONNEI-PHASE

In dem gegen den lebenden Stamm hergestellten Serum wurden die Eigentümlichkeiten des aus der II. Phase von *Sh. sonnei* hergestellten Lipopolysaccharids (Lps) und der ganzen Bakterienzelle untersucht. Ersteres wurde mittels indirekter Hämagglutination, letzteres mit Hilfe der Agglutination studiert. Bei den Erschöpfungsuntersuchungen stellte sich heraus, daß das lebende oder abgetötete Bakterium das agglutinierende Serum völlig erschöpft, während das Lps das Serum nur in bezug auf sich selber erschöpft und die bakteriellen Agglutinine unberührt läßt. Demnach ist das Bakterium-Agglutinogen in der Zelle der II. *Sh. sonnei*-Phase mit dem (somatischen) Lps-Antigen nicht identisch. Zwecks Bestimmung sowie Lokalisierung des Agglutinogencharakters wurde die Zelle verschiedenen chemischen und enzymatischen Wirkungen ausgesetzt. Die Empfindlichkeit des Antigens Lysozym hingegen deutet darauf hin, daß es sich chemisch um ein Acetylglucosaminacetylmuraminsäure-Polymer handelt, da dieses Substrat den Angriffspunkt von Lysozym bildet. Zugleich stellt aber dieses Substrat einen Bestandteil des sog. Weidelschen »R-layer« dar, der unlöslichen Schicht, welche die Festigkeit der Zellwand gewährleistet. Um diese Hypothese bestätigen zu können, bedarf es der Herstellung eines aktiven, isolierten Zellwand-Präparates.

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UNTERSUCHUNG DER DARMFLORA BEI MIT ANTIBIOTIKUM BEHANDELTEN KRANKEN

Die Darmflora von erwachsenen internistischen Kranken wurde untersucht, die mit Erythromycin, Aureomycin sowie peroral und parenteral mit Chloromycetin behandelt worden waren. Nach den Untersuchungsergebnissen

enthält die normale Darmflora in der, ihrem quantitativen Verhältnis entsprechenden Reihenfolge folgende Komponenten: *L. bifidus*, Bacterioides-Genus, *E. coli* und *Str. faecalis*. Akzidentell kommen vor: Clostridium, Klebsiella, Proteus, Staphylokokken, L. acidophylus-Genera, Saccharomyceten, aerobe Sporenträger und anaerobe Streptokokken. Infolge der Antibiotikumbehandlung können sich die permanenten Genera im allgemeinen oder einzelne Genera der Antibiotikumempfindlichkeit entsprechend vermindern bzw. Genera erscheinen oder sich vermehren, die nicht zur permanenten Flora gehören. Diese Veränderungen führen nicht notwendigerweise zu klinischen Beschwerden. Wird in diesen Fällen die Bezeichnung Dysbiosis akzeptiert, so kann auf Grund kritischer Überlegungen dieser Zustand an und für sich nicht als die Ursache pathologischer Veränderungen angesehen werden.

Gy. Biró, H. Milch

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ÜBER DEN WERT DER PHAGTITERERHÖHUNG BEI DER DIAGNOSE EINZELNER ENTERALER KRANKHEITSERREGER

In Übereinstimmung mit den Literaturangaben stellten Verff. fest, daß bei der Diagnostizierung von *Vi*-positiven *S. typhi*-Stämmen die Titererhöhung des entsprechend zusammengestellten *Vi*-Typusphaggemisches gut verwendet werden kann. Sie untersuchten von Reihenuntersuchungen und Kranken stammende Fäzes, künstlich mit *Vi*-positiven *S. typhi*-Stämmen infizierte Fäzes, Trinkwasser und Milch. Die Empfindlichkeit der Reaktion ist im wäßrigen Medium — bei der üblichen Wasserbakteriumflora — wesentlich höher als im Falle der künstlich infizierten Fäzes oder der Milch. Bei letzterer wird die Empfindlichkeit durch Pufferung des Untersuchungsmaterials verbessert. Die Anwendung einer oberflächenaktiven Substanz (Tween 80) in üblicher Konzentration übt keinen Einfluß auf die Ergebnisse aus.

Bei der Untersuchung der unter natürlichen Verhältnissen infizierten Fäzes hat sich die Reaktion bei dem mit Anreicherung verbundenen bakteriologischen Verfahren als empfindlicher erwiesen.

Im Rahmen weiterer Versuche wurde der Nachweis von *S. typhi murium*- sowie *Sh. flexneri*- und *sonnei*-Stämmen mit Hilfe eines spezifischen Phaggemisches untersucht. Die Resultate stimmten mit den oben erwähnten überein.

Die Methode weist den Vorteil auf, daß sie in wesentlich kürzerer Zeit (24—30 Stunden) als die üblichen bakteriologischen Verfahren ein sicher spezifisches, deutlich ablesbares und auswertbares Resultat ergibt. Die Möglichkeit anderweitiger Verwendung der Reaktion wird von den Autoren in weiteren Untersuchungen geklärt.

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DIE ANTIGENSTRUKTUR NEUER ENTEROPATHOGENER COLISTÄMME

Rédey und Csizmazia teilten 1959 mit, daß die Meerschweinchen-Augenreaktion neben den Shigella auch zur Feststellung von Stämmen gut anwendbar sei, die dysenteriforme Erkrankungen beim Menschen hervorrufen. Im Verlauf dieser Untersuchungen gelang es in einigen Fällen, aus der Stuhlprobe von Kranken einen *E. coli*-Stamm zu isolieren, der eine positive keratoconjunctivale Reaktion ergab.

Bei der antigenstrukturellen Analyse erwies sich das O-Antigen des Stammes als 0143.

Neben dem Stamm 0124 ist dies der zweite enteropathogene *E. coli*-Stamm, der die Erkrankung von Erwachsenen hervorzurufen vermag.

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(Veterinärmedizinisches Zentrallaboratorium der Fleischindustrie, Budapest)

ÜBER DEN NACHWEIS DER TIERISCHEN PARATYPHUS VERURSACHENDEN KEIME

Die Entwicklung von 22 *Salmonella cholerae suis* var. Kunzendorf, 15 *S. typhi suis* var. Voldagsen, 17 *S. gallinarum-pullorum*-, 7 *S. abortus equi*-, 7 *S. abortus ovis*- und 2 *S. ab. bovis*-Stämmen wurde auf Kauffmann-, Bierbrauerschen und modifizierten Leifsonschen Selenit-F-Anreicherungsböden, sowie auf Endo-, MacConkey-, Leifson-, SS-, Lovrekovich-, Brillantgrün-Phenolrot-, Gassner-, Schönberg-, Eosin-Methylenblau-Laktose-, Drigalski-, Klimmerschen elektiven und differenzierenden Agarnährböden untersucht. Ferner wurden untersucht die H_2S-, Indolproduktion, der Ureum-Abbau der Stämme, die Voges-Proskauer-Probe, die Methylrot-Reaktion, der Abbau der Sternschen Bouillon, die Entwicklung auf Rhamnose und Trehalose enthaltenden Nährböden, der Abbau von Glukose, Arabinose, Dulcit, Inosit, Rhamnose, Trehalose, Xylose, Mannit, Sorbit, Adonit, Maltose, Salicin, Laktose, die Verflüssigung von Gelatine, die Reduktion von KNO_3, die Bewegung der Stämme und ihr Verhalten gegenüber den Phagen O_1 und R.

Wie festgestellt werden konnte, entwickeln sich einzelne tierische Paratyphuserreger nicht gleichartig auf den Anreicherungs- sowie den elektiven und differenzierenden Nährböden, weshalb Verf. diejenigen Untersuchungsmethoden festzustellen versuchte, die in der praktischen diagnostischen Arbeit unter besonderer Berücksichtigung der Gesichtspunkte der ergänzenden bakteriologischen Fleischuntersuchung mit dem besten Wirkungsgrad angewendet werden können.

T. Fodor, I. Tárnok

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ÜBER DIE RESISTENZ VON MYCOBACTERIUM TUBERCULOSIS GEGENÜBER DIFFUSEM LICHT UND LAUGE

Neuerdings wurde wiederholt bekräftigt, daß die BCG-Bakterien nicht nur durch direktes Tageslicht, sondern auch durch diffuses Licht vernichtet werden. Das *Mycobacterium tuberculosis* enthaltende Untersuchungsmaterial kann ebenfalls von dieser Noxe betroffen werden. Verff. untersuchten die bei kurzwährenden Laboratoriumsarbeiten zur Geltung kommende Lichtwirkung auf die in physiologischer Kochsalzlösung suspendierten Tuberkelbakterien. Ihre Untersuchungsergebnisse fassen sie folgendermaßen zusammen:

1. Von zweistündiger diffuser Lichtwirkung werden die Tuberkelbakterien nicht getötet.

2. Die Lag-Phase der in physiologischer Lösung aufbewahrten Tuberkelbakterien wird verlängert.

3. Die Laugeresistenz der Tuberkelbakterien mit verlängerter Lag-Phase bleibt unverändert.

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IN UNGARN VORKOMMENDE MYKOBAKTERIOSEN

Die von den sog. atypischen anonymen Mykobakterien verursachten Erkrankungen nennt man Mykobakteriosen. Obwohl diese Stämme ausgeprägten Saprophytencharakter besitzen, kommen unter ihnen auch fakultative Krankheitserreger vor. Es gelang, einzelne Stämme zu differenzieren bzw. in eine Spezies einzuordnen.

In Ungarn kennt man bisher zwei Spezies, mit denen gerechnet werden muß: *Mycobacterium minetti* und *Mycobacterium scrophulaceum*. Bei *M. minetti* gelang es nicht, die Pathogenität für den Menschen nachzuweisen, während *M. scrophulaceum* bei Kindern zervikale Lymphadenitis verursachte.

Die bakteriologische und epidemiologische Untersuchung beider Stämme wird ausführlich besprochen.

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DAS VORKOMMEN DER ASCOSPORENFORM VON ACHORION QUINCKEANUM IM BODEN

Bisher kannte man diese Pilzart nur im Conidialzustand und in der vom Wirtstier gezüchteten Form.

Auf feuchten, mit sterilisierten Haaren bestreuten Böden gelang es Verf. 1962, auch das Cleistothecium von *Achorion quinckeanum* anzutreffen. Die aus den Ascosporen auf künstlichen Nährböden gezüchteten Kolonien zeigen die Eigenschaften der Ctenomyces-Arten: sie sind scheibenförmig, weisen im Zentrum einen kleinen Knopf auf, ihre Mikrokulturen erzeugen durchschnittlich 3×4 μ große Conidien, doch kommen unter ihnen (meistens gruppenweise) auch 3—6mal größere und wurstförmige Conidien vor, die einen Übergang zu den Closterosporen bilden. An den Fäden sieht man auch Endosporen, deren Anwesenheit gleichfalls als eine Ctenomyces-Eigenschaft zu werten ist. In den Asci wechselt die Zahl der Sporen: häufig sind 12, mitunter aber auch 24 vorhanden.

Auf Mäuse wirkt die Form kaum pathogen. In den Böden, in denen sich *Achorion quinckeanum* und *Trichophyton gypseum*-Varietäten aufhalten, ist häufig auch *Gymnoascus varticillatus* vorzufinden. Offenbar ist dieser Parasit des im Boden lebenden *Achorion quinckeanum* ebenso wie *Ctenomyces trichophyticus (Trichophyton gypseum)* Parasit der zur Spezies *Achorion gypseum (Microsporum gypseum)* gehörigen. Mit dieser Feststellung erhellt auch die Ernährung der im Boden lebenden Dermatophyten, deren Nahrung weniger aus den fauligen Stoffen der höheren Pflanzen, als aus den Insekten (hauptsächlich Milben) und den im Boden lebenden Fadenpilzen besteht.

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ÜBER DIE EIGENSCHAFTEN DER AUS VERSCHIEDENEN QUELLEN STAMMENDEN ISOLIERTEN STAPHYLOKOKKUS-STÄMME AUF GRUND VON PHAGTYPISIERUNGS- UND ANTIBIOTIKUMEMPFINDLICHKEITSUNTERSUCHUNGEN

Verff. berichten über die Ergebnisse der Phagtypisierungs- und Antibiotikumempfindlichkeitsuntersuchungen, die sie an 4060 zwischen 1960 und 1962 isolierten koagulasepositiven Staphylokokkus-Stämmen durchführten. 434 Stämme stammten von Furunkeln, Abszessen oder Wundsekreten, 234 von pemphigoiden Sekreten, 354 von Ohrensekreten, 2019 von Nasensekreten und 1019 von Rachensekreten.

Bei den von Abszessen, Furunkeln bzw. Wundsekreten stammenden Staphylokokken machten die Stämme der I. Phaggruppe 66% aus; unter diesen betrug das Verhältnis der 80/81 und verwandten Lysisbilder 45%.

Unter den von pemphigoiden Sekreten stammenden Staphylokokkenstämmen dominierte keine einzige Phaggruppe, vielmehr waren sie ziemlich gleichmäßig verteilt.

Eine sehr ähnliche Phaggruppenverteilung wurde unter den von Ohren-, Nasen- und Rachensekreten stammenden Staphylokokken festgestellt: die häufigste Phaggruppe war die III., zu der 33% sämtlicher Stämme gehörten.

Zwischen dem Phagtypus und der Antibiotikumempfindlichkeit wurde eine prägnante Korrelation ermittelt. Während die überwiegende Mehrzahl (80—90%) der zur I. Phaggruppe zählenden Stämme noch mit Chloramphenicol, Tetracyclin, Erythromycin und Neomycin gut beeinflußt werden konnte, erwies sich ein beträchtlicher Prozentsatz (50—60%) der zur III. Phaggruppe rechnenden Stämme nur noch Neomycin gegenüber empfindlich. Die Penicillinresistenz zeigte keine derartige Abweichung: sie war bei den Stämmen sämtlicher Phaggruppen etwa 90%ig.

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UNTERSUCHUNGEN AN KLEBSIELLA-PHAGEN. AUSARBEITUNG EINES TYPUSSCHEMAS

Zur Klebsiella-Gruppe zählende Stämme verschiedener Herkunft wurden von Verff. in Phagtypen eingeteilt. Die zur Typisierung verwendeten spezifischen Klebsiella-Phagen isolierten sie aus dem Stuhl enteritischer Säuglinge. Die isolierten Phagen wurden auf Grund ihrer Plaque-Morphologie, Lysis-Aktivität und serologischen Eigenschaften charakterisiert und in Gruppen eingeordnet.

Die Aufteilung von Klebsiella-Stämmen nach Phagtypen wurde bei den aus dem Stuhl von Frühgeborenen, Säuglingen und zum geringeren Teil von Erwachsenen und aus anderen Sekreten stammenden Stämmen untersucht. Fernerhin ermittelten Verff. das Vorkommen einzelner häufiger Phagtypen anläßlich einer Enteritisepidemie im Krankenhaus sowie bei anderen von Klebsiella-Stämmen verursachten sporadischen Prozessen.

Insgesamt wurden 800 Stämme untersucht, von denen etwa 70% typisiert werden konnten. Es wurde ein Zusammenhang zwischen Phagempfindlichkeit und Pathogenität konstatiert. Die Verff. untersuchten weiterhin die Stabilität der Phagtypen in vivo und in vitro sowie die unter Phagwirkung eintretenden Veränderungen in der Phagempfindlichkeit und im Serotyp.

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ERYSIPELOTHRIX RHUSIOPATHIAE-STÄMME EINES NEUEN SEROTYPS UND DEREN BEDEUTUNG FÜR DIE SERODIAGNOSTIK DER ROTLAUFBAKTERIEN

Der Haptenextrakt von drei aus den Mandeln eines gegen Rotlauf immunisierten Schweines isolierten Rotlaufbakterienstämmen ergab mit den typusspezifischen Sera A_1, A_2, B_1, B_2, C, D und E eine negative Präzipitationsreaktion. Das mit einem der drei Stämme im Kaninchen erzeugte Typusserum präzipitierte nur den Haptenextrakt dieser drei Stämme. Die mit dem Haptenextrakt von Stämmen anderer Serotypen vorgenommene Präzipitationsprobe führte konsequenterweise zu negativen Ergebnissen. Die Stämme agglutinierten nicht die Hühnererythrozyten und erwiesen sich bei intradermaler Infektion als apathogen für Schweine. Auf Grund der Untersuchungsresultate repräsentieren diese Stämme einen neuen Serotyp, der zweckmäßigerweise mit dem Buchstaben G bezeichnet wird.

Aus dem Gesagten geht hervor, daß die säurelöslichen Antigene der Rotlaufbakterien in mehr Varianten vorkommen, als bisher angenommen wurde. Infolgedessen darf man nur denjenigen, sich in S-Kolonieform entwickelnden Rotlaufstamm als N-Typ qualifizieren, von dem man sich überzeugt hat, daß des damit erzeugte Serum auch den homologen Haptenextrakt nicht präzipitiert.

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DIE WIRKUNG DER RÖNTGENSTRAHLUNG AUF DIE BACILLUS CEREUS-SPOREN

Die Strahlentoleranz der vegetativen Zellen, ruhenden sowie keimenden Sporen von *B. cereus* wurde in wäßriger Suspension untersucht und festgestellt, daß die Strahlenresistenz der ruhenden Sporen um nahezu eine Größenordnung höher ist als die der vegetativen Zellen. Die Strahlenempfindlichkeit der keimenden Sporen stimmt im wesentlichen mit der der vegetativen Zellen überein.

Die Keimfähigkeit der ruhenden Sporen wurde auch von den Strahlendosen nicht beeinträchtigt, die sich bei den kulturellen Zählmethoden für lebende Keime als letal wirkend erwiesen. Die Wirkung der ionisierenden Strahlung tritt im Verlauf der postgerminativen Entwicklung zutage.

Phasenkontrastmikroskopische Untersuchungen ergaben, daß hohe Strahlendosen bei den ruhenden Sporen auch im nährmittelfreien Medium eine für die Keimung charakteristische Brechungsindex-Veränderung induzieren. Mit papierchromatographischen, papierelektrophoretischen und spektrophotometrischen Methoden untersuchten Verff. die in Abhängigkeit von der Strahlen-

dosis aus den Mikrobenzellen unter Wirkung der Bestrahlung in das wäßrige Medium ausströmenden Substanzen und den Dipikolinsäuregehalt der Sporen. Nach ihren Feststellungen enthält das Exsudat u. a. Aminosäuren und andere ninhydrinpositive Verbindungen sowie Dipikolinsäure. Die beobachteten Erscheinungen sind zu einem erheblichen Teil auf die unter Strahlenwirkung eintretenden Permeabilitätsveränderungen der Zellen zurückzuführen, deren Dosisbedarf indessen bedeutend größer ist als der der Desorganisierung der die Zellteilung hervorrufenden Prozesse.

Die Untersuchung der Dehydrogenase-Aktivität der bestrahlten Mikroorganismen führte zu dem Ergebnis, daß die Enzymtätigkeit bei den »toten« (sich nicht vermehrenden) Zellen nicht aufhört. Bei der Sterilisierung von Lebensmitteln durch Bestrahlung muß demnach nicht nur mit der großen Strahlenresistenz der Gewebsenzyme, sondern auch mit einer auch in sterilen Produkten eintretenden gewissen »mikrobialen Enzymtätigkeit« gerechnet werden.

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EINFACHE NÄHRBÖDEN ZUR ZÜCHTUNG VON OCHROMONAS MALHAMENSIS

Das Protozoon *Ochromonas malhamensis* stellt den spezifischsten Testorganismus zur Bestimmung von Vitamin B_{12} dar. Seiner Anwendung setzt jedoch der Umstand eine Schranke, daß der für seine Züchtung vorgeschriebene Nährboden eine sehr komplizierte Zusammensetzung aufweist bzw. zahlreiche reine Salze und Vitamine des B-Komplexes enthält.

Zur Züchtung von *Ochromonas malhamensis* empfiehlt Verf. zwei einfache Nährböden. In dem einen wird der Vitamin B-Komplex mit Tomatensaft ersetzt, im anderen ein methanol-azetonhaltiger Maismarmeladeextrakt benutzt, der nicht nur den Vitaminkomplex, sondern zugleich auch das Kaseinhydrolysat substituiert. Dieser Maismarmeladeextrakt eignet sich auch in anderen Nährböden zum Ersatz von Kaseinhydrolysat.

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BIOCHEMISCHE UNTERSUCHUNG DER PEPTONWASSER-ALKALISIERUNGSREAKTION VON BAKTERIEN

Wie Serény 1963 mitteilte, wird von den in kochsalzhaltiges Peptonwasser geimpften und bei 37° C inkubierten Darmbakterien der Nährboden alkalisiert. Da man sich den Alkalisierungsmechanismus auf verschiedene

Weise vorstellen kann, führten Verff. Untersuchungen zur Klärung dieser Frage durch.

Nach ihren Feststellungen beruht die Alkalisierung des Peptonwassers auf Ammoniakbildung. Zugleich mit der Ammoniakbildung verschwinden Aminosäuren aus dem Nährboden. Auf Grund dieser Beobachtung wurde versucht, Pepton mit singulären Aminosäuren zu substituieren. In den singulären Aminosäurelösungen konnten die Ammoniakfreisetzung und der Aminosäurenverbrauch gleichfalls nachgewiesen werden.

Nach den Beobachtungen werden die Aminosäuren von den Bakterien im Peptonwasser bzw. in der Aminosäurelösung als Atmungs- und Synthesesubstrat verbraucht, und als Nebenprodukt dieses Prozesses wird das Ammoniak freigesetzt. Der gemeinsame Kohlen- bzw. Kohlenverbindungsbedarf der Energiefreisetzung und der synthetisierenden Prozesse ist größer als der Stickstoffbedarf der synthetisierenden Prozesse, so daß das Ammoniak bei einem Aminosäurensubstrat aus dem Stickstoffüberschuß der Aminogruppen stammt. Das Ammoniak entsteht durch oxydative Desaminierung aus der Aminosäure, doch handelt es sich bei dem Primärprozeß um Dekarboxylierung.

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ZWEISCHICHTIGER PLATTENNÄHRBODEN ZUR BIOCHEMISCHEN DIFFERENZIERUNG DER KEINEN SPEZIELLEN NÄHRBODEN BEANSPRUCHENDEN BAKTERIEN

Ein neuer Nährboden zur Untersuchung von Darmbakterien wurde ausgearbeitet, der sich zur Untersuchung der Spaltung von 10—11 verschiedenen Kohlenhydraten, zum Nachweis der entstandenen Säuren und Gase sowie zur Bestimmung der Schwefelwasserstoffbildung, der Ureumspaltung, des Gelatineabbaus, der Indolbildung sowie der Methylrot-Reaktion eignet. An der Oberfläche des Nährbodens kann die serologische Untersuchung mit der Bakterienvegetation vorgenommen werden.

Mit Hilfe des Nährbodens vermag man die Kohlenhydratspaltung der Darmbakterien an Hand der Säure- und Gasbildung, des Ureumabbaus, der Indolbildung und der Schwefelwasserstoffproduktion binnen 6—7 Stunden zu bestimmen. Die anderen Untersuchungen können nach 18—20 Stunden durchgeführt werden. Die Inkubation bei 37° C dauert 6—7 Stunden, wonach der erste Teil der Auswertung erfolgen kann; anschließend 3 Stunden Zimmertemperatur, wonach die Kultur 18—20 Stunden gleichfalls bei Zimmertemperatur aufbewahrt und nunmehr die Auswertung vorgenommen wird.

Beim Vergleich mit den klassischen Methoden ergab die Methode entsprechende Resultate, so daß sie sich zur biochemischen Untersuchung und Differenzierung von Darmbakterien eignet.

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UNTERSUCHUNGEN ZUR RASCHEN UND GENAUEN BESTIMMUNG VON KEIMZAHLEN

Die rasche und präzise Bestimmung von Keimzahlen kommt sowohl in der theoretischen, wie als auch in der praktischen mikrobiologischen Arbeit zur Bedeutung. In gewissen Fällen ist die verhältnismäßig lange Inkubationszeit der Züchtungsmethoden nachteilig. Zwecks Vermeidung dieses Nachteils wendete Verf. — neben biochemischen Methoden — im Falle einiger Bakterien- und Hefearten die auf der Impulstechnik beruhende Zellzahlbestimmung an.

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DIE MIKROBIOLOGISCHE UNTERSUCHUNG UND BLUTDRUCKSENKENDE WIRKUNG DES »JAPANISCHEN PILZES«

Der sog. »japanische Pilz« wird als Volksheilmittel gegen hohen Blutdruck benutzt. Hierbei handelt es sich nicht um eine einheitliche Substanz, sondern um einen auf Grund der Symbiose von Hefe und Essigsäurebakterien gebildeten Belag, den man in gezuckertem Tee zu züchten pflegt. Aus diesem »japanischen Pilz« konnten verschiedene Mikroorganismen isoliert werden: eine Hefeart und mehrere Bakterien. Die pharmakologischen Untersuchungen führten zu folgenden Resultaten:

1. Die Bakterienkultur enthält keine blutdrucksenkende Substanz.
2. In der Hefekultur ist ein blutdrucksenkender Stoff enthalten.
3. Der blutdrucksenkende Stoff ist an die Hefezellen gebunden; der zellfreie Nährboden enthält diesen Stoff nicht.
4. Die blutdrucksenkende Substanz ist thermostabil.
5. Die blutdrucksenkende Substanz kann auf verschiedene Weise aus den Zellen extrahiert werden. Unter den erprobten Methoden hat sich das Kochen mit verdünnter Essigsäure am wirksamsten erwiesen.
6. Zu Vergleichszwecken wurde der blutdrucksenkende Effekt auch anderer Hefekulturen untersucht und festgestellt, daß auch andere Hefearten einen gewissen blutdrucksenkenden Stoff enthalten, aber die blutdrucksenkende Wirkung z. B. von Backhefe 4—5mal geringer ist. Zwei andere wilde Hefestämme wirkten gleichfalls schwächer.
7. Die blutdrucksenkende Wirkung des »japanischen Pilzes« — insbesondere die der reinen Hefekulturen — ist ziemlich intensiv, aber nicht anhaltend, so daß er den Wettbewerb mit den modernen blutdrucksenkenden Medikamenten nicht aufzunehmen vermag.

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DIE WIRKUNG DER BEI MASTITIS GEWONNENEN MILCH AUF DIE LEBENSFUNKTIONEN DER MILCHSÄUREBAKTERIEN IN DER BUTTERKULTUR

Sekretorisch fehlerhafte Milchproben wurden (nur in subklinischen Fällen) mit der als zuverlässig angesehenen Whiteside-Probe selektiert und die verschiedenartig wärmebehandelten (rohen, bei 75, 85 und 100° C 1 Minute pasteurisierten sowie sterilen) Whiteside-positiven und -negativen Milchproben gesondert mit Butterkultur sowie *Str. lactis*- und *Str. cremoris*-Reinkulturen beimpft.

Wie festgestellt wurde, geht die Säureerzeugung der Milchsäurebakterien in der als negativ (gesund) bezeichneten Milch mit genügender Intensität vor sich; der definitive Säuregrad, die Aromareaktion und die physikalischen Eigenschaften der Kultur weisen die gewünschten Charakteristika auf. In der Whiteside-positiven Milch erfolgt die Säureproduktion langsamer, der Endsäuregrad blieb stets unter 30° SH. Die Aromareaktion der aus dieser Milch hergestellten Kultur ergibt — oder ±, ihr Geschmack ist in der Regel herb, bitter, unangenehm fremd, widerlich, nicht rein, ihre Konsistenz ist weich, flockig, gashaltig.

Die Gesamtkeimzahl der aus der rohen und verschiedenartig wärmebehandelten gesunden Milch zubereiteten Kultur ist 3—9mal höher als die der aus positiver Milch hergestellten Kultur. Der Unterschied im Endsäuregrad und in der Keimzahl der beiden Kulturen läßt sich wahrscheinlich darauf zurückführen, daß die Vermehrung der Milchsäurebakterien von der sekretorisch fehlerhaften Milch gehemmt wird. Einige ausländische Forscher schrieben die Hemmwirkung der bei Mastitis gewonnenen Milch dem Mangel der den sog. antibakteriellen Faktoren entsprechenden Wachstumsstoffe, der höheren Leukozytenzahl und der antagonistischen Wirkung der die Euterentzündung verursachenden Streptokokken zu.

Auf Grund eigener Untersuchungen ist es wahrscheinlich, daß die Hemmwirkung nicht auf den Stoffwechselprodukten der Entzündung hervorrufenden Streptokokken und auf der hohen Leukozytenzahl beruht, sondern in erster Linie auf der infolge veränderter Zusammensetzung niedrigeren Pufferkapazität der sekretorisch fehlerhaften Milch, auf der Anwesenheit von wärmeempfindlichen und thermostabilen Antikörpern sowie vermutlich auf dem Mangel einzelner wichtiger Biostoffe.

Die mittels Säuregradbestimmung kontrollierte Vermehrungshemmung hörte in der 5% mastitische Milch enthaltenden Kultur auf, während die schädliche Wirkung der sekretorisch fehlerhaften Milch auf den Aromagehalt, Geschmack und die Konsistenz der Kultur sich auch bei niedrigerem Prozentsatz manifestierte.

VIROLOGIE

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UNTERSUCHUNG DER ENTEROVIREN VON SCHWEINEN

Aus den Kotproben von 58 hauptsächlich aus großen Schweinebeständen stammenden gesunden und unter verschiedenen klinischen Symptomen erkrankten Schweinen züchtete Verf. 185 Schweine-Enterovirus-Stämme (ECSO) in isolierten Schweinenierengewebskulturen. Mit Hilfe von 37 aus Kaninchen erzeugten Immunsera reihte er die Stämme in mindestens 16 serologische Gruppen ein. Von diesen geben die Stämme einer Gruppe Kreuzreaktion mit dem infektiösen (Teschener) Schweinelähmungs-Virus. Auf Grund des morphologischen Bildes der in den Gewebskulturen entstandenen zytopathologischen Veränderungen bilden die Stämme zwei Typen, deren Bestimmung auch in nativen Kulturen möglich ist und die mit der serologischen Gruppierung der Stämme zusammenhängende Arbeit erleichtert. Auf colostrumfrei künstlich aufgezogene junge Ferkel wirken einzelne Stämme pathogen.

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VERSUCHE ZUR REINIGUNG VON HERPES SIMPLEX VIRUSSUSPENSIONEN

Die Behandlung von Herpes simplex-Virussuspension mit ansteigenden Rivanolmengen ergab, daß das infektive Virus größtenteils mit einer geringen Rivanolmenge ausgefällt werden kann, die den überwiegenden Teil des Eiweißgehaltes der Virussuspension noch in Lösung beläßt. Aus dem Niederschlag kann man das Virus in infektiver Form zurückgewinnen.

Wird das auf HeLa-Zellen gezüchtete Herpes simplex-Virus auf die DEAE-Zellulosesäule übertragen und mit einer ansteigende Kochsalzkonzentrationen enthaltenden Pufferlösung eluiert, so ist festzustellen, daß der Eiweißgehalt der Virussuspension größtenteils mit der Pufferlösung von niedrigerer Konzentration, dagegen das infektive Virus mit der Pufferlösung von höherer Kochsalzkonzentration eluiert werden kann.

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ISOLIERUNG VON BOVIN-ADENOVIRUS IN KALBSHODENZELLKULTUREN

Aus dem Kot sowie Nasensekret gesunder Kälber, weiterhin aus der Lunge eines an virusbedingtem Durchfall eingegangenen Kalbes konnte in Kalbshodenzellkulturen ein Virus isoliert werden, das auf Grund seiner physikalischen und zytopathogenen Eigenschaften zur Gruppe der Adenoviren rechnet. Die mit dem isolierten Virus infizierten Kälber und Laboratoriumsversuchstiere erkrankten nicht.

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ÜBER DIE SPEZIFISCHEN ANTIGENEIGENSCHAFTEN DER EINSCHLUSSKÖRPERCHEN BEI KERATOCONJUNCTIVITIS EPIDEMICA

In den Jahren 1961—62 ist in Ungarn eine Keratoconjunctivitis epidemica-Epidemie aufgetreten. In 88% der aus der akuten Krankheitsphase stammenden Bindehautabstriche enthielten die Epithelzellen charakteristische Einschlußkörperchen. In Immunfluoreszenzuntersuchungen wurde festgestellt, daß diese Einschlußkörperchen spezifische Antigeneigenschaften besitzen, die der Antigennatur des Adenovirus 8 entsprechen, das auch bei dieser Epidemie als Haupterreger zu betrachten war. Zwecks Nachprüfung der Antigenspezifität der Einschlußkörperchen wurden die solche sicher enthaltenden Bindehautepithelzellen zur Kontrolle mit Adenovirus-Immunsera der Typen 3, 4, 5, 6, 7 und 11 behandelt, ohne daß charakteristische Fluoreszenz in irgendeinem Fall festgestellt werden konnte.

Die Immunfluoreszenzuntersuchungen zahlreicher, von anderen Bindehautentzündungen stammender Abstriche ergaben mit dem Adenovirus 8 gegenüber erzeugten Immunserum gleichfalls ein negatives Resultat.

Der von der Bindehaut der an Keratoconjunctivitis epidemica leidenden Kranken in 122 Fällen entnommene Abstrich bestätigte im Vergleich zu den Kontrollen überzeugend, daß die Einschlußkörperchen bei Keratoconjunctivitis epidemica die pathogenen Virusteilchen teilweise oder ganz enthalten.

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DIE WIRKUNG DES MODIFIZIERTEN FRANCIS-INHIBITORS AUF DIE VERMEHRUNG DES INFLUENZA-VIRUS

Von der aus dem modifizierten Francis-Inhibitor hergestellten Fraktion wird die Vermehrung des Influenza-Virus im deembryonierten Ei sehr beträchtlich gehemmt. Mit einer zytotoxischen Wirkung läßt sich die Erscheinung nicht erklären, weil die Vermehrung des Vaccinia-Virus unter gleichen Bedingungen nicht gehemmt wird. Der Effekt auf die in der Allantoismembran vor sich gehende Vermehrung findet hauptsächlich zu Beginn der Ekliptenphase und dann statt, wenn die Fraktion bei der Virusfreisetzung zugegeben wird. Die Ausscheidung der bereits reifen Partikelchen aus der Zelle wird beschleunigt. Die in der Nährflüssigkeit wahrgenommene hochgradige Hemmwirkung hängt mit dem viruziden Charakter der Substanz zusammen. Da es sich bei der fraglichen Substanz um ein Zellrezeptor-Analogon handelt, muß der Zellrezeptor nicht nur in der Früh-, sondern auch in der Spätphase der Virusvermehrung eine Rolle spielen.

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INFEKTION VON IM NEUGEBORENENALTER THYMEKTOMIERTEN MÄUSEN MIT LCM-VIRUS

Die Untersuchungen wurden an nicht aus Inzucht stammenden weissen und CBA-Mäusen vorgenommen. Nach ic. Infektion von 4—5 Wochen alten, im Neugeborenenalter thymektomierten Mäusen mit einer massiven LCM-Virusdosis wurde der theoretischen Hypothese entsprechend verminderte Empfänglichkeit dem Virus gegenüber festgestellt, die sich in einer verlängerten Inkubationszeit bzw. darin manifestierte, daß $^1/_3$ der Mäuse mehr als 14 Tage (14—49 Tage) überlebte. Die mit diesen Mäusen auf derselben Streu gehaltenen Kontrolltiere gingen nach der üblichen Inkubationszeit (6—7 Tage) ein. Bei der Sektion der nach prolongierter Inkubationszeit verendeten Versuchstiere wurde festgestellt, daß in etwa 20% der Fälle die Thymusexstirpation vollwertig war und die Verlängerung der Inkubationszeit im Verhältnis zur Radikalität der Operation stand. Bei einer Gruppe von thymektomierten, aber nicht mit LCM-Virus infizierten Mäusen konnten die klinischen und histologischen Symptome der »wasting disease« wahrgenommen werden.

Aus den Ergebnissen wurde die Schlußfolgerung gezogen, daß die LCM-Virusinfektion ein geeignetes Modell zur Untersuchung des Problems der Thymusexstirpation im Neugeborenenalter darstellt.

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UNTERSUCHUNG DER VON EINEM NICHT ZYTOPATHOGENEN SCHWEINEPEST-VIRUSSTAMM HERVORGERUFENEN ZYTOCHEMISCHEN VERÄNDERUNGEN UND DEREN BEDEUTUNG FÜR DIE ERKENNUNG DER EIGENSCHAFTEN DES VIRUS

Mittels Immunfluoreszenz- und zytochemischen Untersuchungen wurden die unter Wirkung der nicht zytopathogenen Schweinepest-Virus-Infektion in primären, einschichtigen Schweineembryo-Nierenepithelzellkulturen vor sich gehenden Ereignisse beobachtet und das intrazelluläre Erscheinen des Virusantigens verfolgt. Die Untersuchungen wurden mit einem in der 6—7. Gewebskulturpassage befindlichen Schweinepest-Virusstamm vorgenommen, der sich — nach dem Immunfluoreszenzverfahren untersucht — in der Verdünnung 10^{-4} als infektionsfähig erwies.

48 Stunden nach der Infektion konnten in den mit Hämatoxylin-Eosin, nach Giemsa und mit kolloider Sudan-Lösung gefärbten Präparaten im Vergleich zu den nicht infizierten Kontrollen keine Veränderungen nachgewiesen werden. Die Feulgen-Reaktion ergab ein negatives Resultat. Im Zytoplasma der mit chrom- und alaunhaltigem Gallocyanin gefärbten Zellen waren auf Nukleinsäurevermehrung deutende Anzeichen zu beobachten, dementsprechend trat in den mit Acridinorange gefärbten Präparaten eine Ribonukleinsäureanhäufung anzeigende Fluoreszenz zutage. Die auf diese Weise nachgewiesene Ribonukleinsäure hat der Ribonukleasebehandlung nicht widerstanden. Die Identität mit dem Virusantigen wurde in Immunfluoreszenzuntersuchungen erhärtet.

Auf Grund der Ergebnisse wird unter Berücksichtigung der schon zuvor bekannten Eigenschaften angenommen, daß das Schweinepest-Virus zu den ribonukleinsäurehaltigen, äther- und chloroformresistenten, 27—30 mμ großen, kugelförmigen Viren zählt und infolgedessen alle jene Kriterien aufweist, auf Grund welcher seine Einordnung in die Picorna-Virusgruppe angezeigt erscheint.

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ÄTIOLOGISCHE UNTERSUCHUNG VON EPIDEMIOLOGISCHEN UND SPORADISCHEN KERATOCONJUNCTIVITIS-ERKRANKUNGEN

Von November 1961 bis April 1962 wurden 330 Untersuchungsmaterialien von Personen, die an Keratoconjunctivitis epidemica erkrankt waren, in Virusisolierungsversuchen aufgearbeitet. Mit dem Serumpaar von 160 Kranken wurden zum Nachweis der Adenovirusinfektion Komplementbindungs- und

Hämagglutinationshemmungsuntersuchungen vorgenommen. Während die Zahl der isolierten Virusstämme zu gering war, um konkrete Schlußfolgerungen ziehen zu können, deuteten die serologischen Methoden, vor allem die HA-Hemmung, in 50% der Fälle übereinstimmend auf die pathogene Rolle des Adenovirus 8.

Im Verlauf der zwischen August 1962 und Juni 1963 sporadisch aufgetretenen Keratoconjunctivitis-Erkrankungen wurde das Untersuchungsmaterial von 32 Kranken aufgearbeitet.

Aus den Virusisolierungsversuchen und Antikörperuntersuchungen ging die pathogene Rolle verschiedener Viren (Adenovirus 3, 4, 8, 14, Herpes simplex-Virus) hervor.

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INTERFERENZ ZWISCHEN HERPESVIRUS SUIS UND HERPESVIRUS HOMINIS IN HeLa-ZELLEN

Nach Infektion mit dem Herpesvirus suis (AY-Virus) in der Multiplizität 1—0,1 ist keine Viruswirkung an HeLa-Zellen wahrnehmbar. Am 3—4. Tage nach der Infektion erwiesen sich die Zellen dem Herpesvirus hominis gegenüber ausgesprochen immun, d. h. sie waren der 100—1000 CPD 50-Virusmenge gegenüber resistent. Gleichzeitig waren die Zellen dem Poliomyelitis-Virus 2, dem Adenovirus 3 und Parainfluenza-Virus 3 gegenüber ebenso empfindlich wie die Kontrollzellen. Zwecks Klarstellung des Mechanismus der spezifischen, nur mit dem Herpesvirus hominis vorhandenen Interferenz wurden weitere Versuche ausgeführt. Hierbei konnte nach Infektion mit dem AY-Virus keine Interferon-Erzeugung nachgewiesen werden. Das mit UV-Licht inaktivierte AY-Virus interferierte nicht mit dem Herpesvirus hominis. Die Untersuchungsergebnisse gestatten den Schluß, daß sich eine intrazelluläre, nicht auf Interferonwirkung beruhende Interferenz spezifisch zwischen Herpesvirus suis und Herpes hominis entwickelt.

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VERGLEICHENDE UNTERSUCHUNG DER ANTIGENSTRUKTUR UND ZYTOPATHOGENEN EIGENSCHAFTEN EINHEIMISCHER HERPESVIRUS HOMINIS STÄMME

Mit Hilfe der an HeLa-Zellen bewerteten Virusneutralisationsproben wurde die Antigenidentität von 11 einheimischen Stämmen im Verhältnis zueinander und zu zwei ausländischen Stämmen untersucht.

An HeLa-Zellen zeigten die Stämme keine einheitlichen zytopathogenen Eigenschaften. Rundzellige Zelldesintegration, Synzytiumbildung oder beide zytopathogenen Wirkungen waren gemischt zu beobachten. Die rundzellige Desintegration ergebenden Stämme führten in einzellschichtigen Hühnerembryokulturen zur Bildung von Mikro-Plaques, die synzytiumsteigernden Stämme zur Bildung von Makro-Plaques. Fernerhin wurden zur Klarstellung der Rolle einzelner Faktoren Untersuchungen über die Entwicklung der verschiedenen zytopathogenen Eigenschaften durchgeführt.

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ISOLIERUNG DES PARAINFLUENZA-VIRUS 3 IN DER GEWEBSKULTUR NACH EINEM MODIFIZIERTEN VERFAHREN

Die geringe Pathogenität des Parainfluenza-Virus 3 HeLa-Zellen gegenüber ist bekannt. Im Verlauf von Virusisolierungsversuchen fiel an den mit der Rachenspülflüssigkeit eines Kindes infizierten Zellen die am 18. Tage der Inkubation vorgenommene Hämadsorbtionsuntersuchung positiv aus. Hiernach wurden mehrmals frische HeLa-Kulturen mit der Flüssigkeit der Kulturen infiziert, es gelang jedoch nicht, das Virus weiterzuimpfen. Am 30. Tage der Inkubation konnte mit Hilfe der mittels Trypsinverdauung hergestellten Zellpassage der beimpften Zellkultur das Virus isoliert werden, das sich im Verlauf der weiteren Untersuchungen als Parainfluenza 3-Virus erwies. Die mit dem Serumpaar des Kranken ausgeführten Neutralisationsproben bestätigten den Zusammenhang des auf diese Weise isolierten Virus mit der fraglichen Erkrankung.

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VON VIREN DER PSITTACOSIS-LYMPHOGRANULOMA-VENEREUM-(P-LGV)GRUPPE VERURSACHTE SCHAF- UND KÄLBERERKRANKUNGEN

In mehreren Schafzuchtbeständen kam größtenteils gleichzeitig mit den virusbedingten Aborten (Stamp-Virus) unter den gemeinsam mit den Muttertieren, den jungen Lämmern, aber auch mit den trächtigen Tieren gehaltenen Jungtieren eine katarrhalische Lungenentzündung vor. Aus den Lungen gelang es, P—LGV-Virusarten im Dotter von 6—8 Tage alten Hühnerembryos zu isolieren. Diese Tatsache einerseits sowie der durch die Luftröhre vorgenommene erfolgreiche Infektionsversuch mit dem aus der Fruchtblase der abortierten Schafe gezüchteten einheimischen Virus anderseits scheint die Hypothese zu bekräftigen, daß die aus den katarrhalischen Lungenentzündun-

gen und den Aborten hier isolierten P—LGV-Viren einander nahestehen oder identisch sind. Die Lungenentzündung ist somit als eine Manifestationsform dieser Virusinfektion zu betrachten.

Von den in Kalbszüchtereien plötzlich in akuter Form auftretenden Bronchopneumoniefällen gelang es, einige Virusarten der P—LGV-Gruppe zu züchten. Mit dem im Dottersack von 6—7 Tage alten Hühnerembryos haftenden Virus konnten junge Kälber intravenös und intratracheal erfolgreich infiziert werden.

Im Blutserum der Schafe und Kälber, welche die Krankheit überstanden hatten, war die Antikörpermenge bei den mit den thermostabilen Antigenen der P—LGV-Viren durchgeführten Komplementbindungsproben charakteristisch vermehrt.

Bei den jungen Kälbern, die mit dem aus der Lammlunge isolierten Virus intratracheal infiziert wurden, waren auf die Krankheit hinweisende leichte Symptome und Veränderungen zu beobachten. Die mit dem von Kälbern gezüchteten Virus infizierten Lämmer wiesen eine fast 41° C erreichende Temperaturerhöhung auf, und ihre Lunge zeigte makroskopische und histologische Veränderungen. Dies beweist, daß im Zusammenhang mit den P—LGV-Viren von strenger Artspezifität keine Rede sein kann.

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ÜBER DIE ISOLIERBARKEIT VON ENTEROVIREN BEI ERKRANKUNGEN DES ZENTRALNERVENSYSTEMS VOR (1958—59) UND NACH (1960—62) DER EINFÜHRUNG DER LEBENDEN POLIOMYELITIS-VAKZINE IN UNGARN

Die Ergebnisse der in den Jahren 1958—1962 durchgeführten Enterovirus-Isolierungsversuche bei den an Poliomyelitis, an poliomyelitisartigen Erkrankungen sowie an enzephalitischer Fazialisparese und an aseptischer Meningitis Erkrankten werden von Verff. hauptsächlich von dem Gesichtspunkt aus analysiert, ob sich die Isolierungshäufigkeit der verschiedenen Enteroviren seit Einführung der Impfungen mit lebender Poliomyelitis-Vakzine (1960) im Vergleich zur vorangegangenen Periode (1958—59) geändert hat.

In den Jahren 1958—59 erwiesen sich 78% von 635 Poliomyelitiskranken als Enterovirusausscheider (75% Polio, 1% Coxsackie, 2% ECHO), während in den Jahren 1960—62 von den auf klinisch als Poliomyelitis erklärten 43 Fällen 46% Virus ausschieden (44% Polio, 2% Coxsackie). Von den an anderen neuralen Erkrankungen Leidenden wurden 864 in der ersten und 1181 in der letzteren Periode untersucht. Die Virusausscheidungshäufigkeit betrug 1958—59 23% (10% Polio, 8% Coxsackie, 5% ECHO), 1960—62 dagegen 10%

(3% Polio, 2% Coxsackie, 5% ECHO). In den Jahren 1960—62 wurde Poliovirus ungeachtet des Krankheitsbildes ausschließlich im Verlauf der ersten Halbjahre, d. h. in den Vakzinationsperioden, dagegen 1958—59 bei Poliomyelitiden im ganzen Jahr und bei den anderen Krankheitsbildern in der zweiten Jahreshälfte isoliert. Diese Ergebnisse bestätigen, daß die Zirkulation der wilden Polioviren seit 1960 in Ungarn praktisch aufgehört hat. Die Häufigkeit des Vorkommens anderer Enteroviren bei den Erkrankungen des Zentralnervensystems hat sich seit Einführung der lebenden Polio-Vakzine nicht geändert.

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DIE HUMORALE IMMUNITÄT GEGENÜBER POLIOMYELITIS DER UNGARISCHEN BEVÖLKERUNG IM APRIL 1963

Zwecks Bestimmung des humoralen Immunitätsgrades gegenüber Poliomyelitis wurden im April 1963 aus 24 Verwaltungsbezirken von etwa 40‰ der Bevölkerung sämtlicher Altersgruppen Blutproben entnommen. Lediglich bei 202 der untersuchten 3934 Personen (5,1%) wurde Antikörpermangel irgendeinem Poliovirus-Typ gegenüber festgestellt. Von diesen besaßen 63 (1,6%) keine Antikörper Polio 1, 46 (1,2%) Polio 2, 74 (1,9%) Polio 3, 10 (0,3%) Polio 1 + 3, 8 (0,2%) Polio 2 + 3 gegenüber, während 1 Person keinem Typ gegenüber Antikörper aufwies. Das Verhältnis der seropositiven Personen sämtlichen drei Typen gegenüber erreichte oder überstieg 95% in sämtlichen Altersgruppen, die im Jahre 1962 Geborenen ausgenommen. Bei den Männern war das Verhältnis der Seronegativen etwas höher als bei den Frauen. Im Immunitätsniveau der in verschiedenen Verwaltungsbezirken lebenden erwachsenen ungeimpften Bevölkerung traten keine wesentlichen Unterschiede zutage. Diese Tatsache sowie der Umstand, daß die Mehrzahl der Ungeimpften einen hohen Antikörperspiegel aufwies, gestatten die Schlußfolgerung, daß die Streuung der attenuierten Poliovirusstämme anläßlich der jährlich wiederholten Impfkampagnen ausreicht, um die Immunität der Gesamtbevölkerung auf einem entsprechenden Niveau zu halten.

BAKTERIELLE BIOCHEMIE

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GASCHROMATOGRAPHISCHE ANALYSE DER FETTSÄUREN VERSCHIEDENER BAKTERIEN

Die Feststellung des Fettsäurespektrums der verschiedenen Bakterien ermöglicht die genauere systematologische Einordnung der einzelnen Bakterien sowie die präzisere Bestimmung bisher weniger untersuchter Phasen des Bakterienstoffwechsels. Mit Hilfe dieser Methode untersuchten Verff. das Fettsäurespektrum sowie die qualitative und quantitative Zusammensetzung von 6 verschiedenen Bakterien-Spezies.

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ERFAHRUNGEN BEI DER CHEMISCHEN UNTERSUCHUNG VON LUES-ANTIGENEN

Im Verlauf der Schicht- und gaschromatographischen Analyse können charakteristische Unterschiede unter den Komponenten der einzelnen Antigene sowie in ihrem Gehalt an gesättigten und ungesättigten Fettsäuren nachgewiesen werden. Im Zusammenhang mit diesen Untersuchungen wird auf die Bedeutung dieser Komponenten bei der Entwicklung der Komplementbindungs- und Präzipitations-Luesreaktionen hingewiesen.

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UNTERSUCHUNG DER INDUZIERENDEN WIRKUNG VERSCHIEDENER ZYTOSTATISCHER MITTEL AN MEGAZINOGENEN UND LYSOGENEN BACILLUS MEGATERIUM-STÄMMEN

Die Wirkung von insgesamt 14 in Verkehr befindlichen und auch klinisch angewendeten zytostatischen Mitteln auf den megazinogenen *B. megaterium*-Stamm Nr. 216 sowie auf den lysogenen *B. megaterium*-Stamm Nr. 899 wurde untersucht. Diese Stämme lösen sich unter Wirkung von UV-Bestrahlung unter Produktion von Megazin bzw. Phagen auf. Drei Mittel, Mitomycin C, Trenimon und Carcinophyllin (Kyowa), ahmten vollkommen die Wirkung der UV-Strahlen nach, d. h. sie führten zur Megazin- bzw. Phaginduktion. Als wirkungslos auf den megazinogenen Stamm erwiesen sich: Stickstofflost,

Betaphenyl-äthylalkohol, Degranol, 6-Mercaptopurin, Sarcolysin (Actinomycin C) sowie das bekanntermaßen mutagen wirkende Sulfonat. Bei den untersuchten Substanzen handelt es sich sowohl chemisch, als auch in ihren bisher bekannten Wirkungen um sehr verschiedene Stoffe. Ihr angeblicher Antitumoreffekt kann auch schon auf dieser Grundlage auf sehr verschiedenen Wirkungsmechanismen beruhen. Ein Teil von ihnen wirkt auf die DNA-Synthese, doch tritt die Hemmung der Nukleinsäuresynthese verschiedenartig zutage. Die Wirkung der die Induktion hervorrufenden Substanzen erscheint als ein typisch radiomimetischer UV-Effekt. Die mutagene Eigenschaft ist von der Induktionswirkung unabhängig, weil z. B. Äthylmethansulfonat keine induzierende Eigenschaft zeigte. Von Betaphenyl-äthylalkohol wird die DNA-Synthese gleichfalls gehemmt, was aber mit der Trennung des DNA-Doppelfadens zusammenhängt. Es scheint, daß das Studium der Megazin-Induktion bei der Screening und Gruppierung der zytostatischen Substanzen Hilfe zu leisten vermag.

IMMUNOLOGIE

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ÜBER DIE BEZIEHUNGEN ZWISCHEN DER STRUKTUR UND FUNKTION DER IMMUNGLOBULINE

(Zusammenfassung nicht eingegangen)

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ERZEUGUNG GEWEBSFIXIERTER (SESSILER) ANTIKÖRPER IN MEERSCHWEINCHEN UNTER WIRKUNG EINES EINMALIGEN ANTIGENSTIMULUS

40 Meerschweinchen wurden mit 1,3 mg/100 g Jodovalbumin sensibilisiert und hiernach in Intervallen von 1—3 Tagen jeweils 1 Tier getötet. Mit dem Serum wurde die quantitative Präzipitation vorgenommen bzw. in Leber und Lunge die Menge der sessilen Antikörper nach der früher beschriebenen, auf Haptenhemmung beruhenden Isotopmethode bestimmt. Zur Antigenmarkierung wurde J^{131} benutzt.

Nach den Ergebnissen enthält das Serum der sensibilisierten Meerschweinchen kreisende Antikörper nur in minimaler Menge: etwa 3 Wochen nach der Sensibilisierung können 10—30 μg Antikörper—N/ml nachgewiesen werden. Demgegenüber enthält sowohl die Leber, als auch wie die Lunge bereits 24 Stunden nach der Sensibilisierung eine meßbare Menge sessiler Antikörper.

Der Antikörpergehalt dieser organe erreicht den ersten Höhepunkt 5—7 Tage nach der Sensibilisierung, sodann nimmt ihre Menge vorübergehend ab, wonach sie am 18—22. Tage im Vergleich zum ersten Maximum bedeutend höher ansteigt. Vom 25. Tage an nimmt der sessile Antikörpergehalt der Leber und Lunge ab, doch können auch noch am 50. Tage gut meßbare Mengen nachgewiesen werden.

Auf Grund dieser Bestimmungen wird verständlich, warum unter den Laboratoriumsversuchstieren das Meerschweinchen das geeignetste Modellobjekt für die experimentelle Anaphylaxie bildet und warum nach einmaliger Sensibilisierung der schwerste anaphylaktische Schock am 18—22. Tage ausgelöst werden kann.

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VERSUCHE ZUR VERÄNDERUNG DER ARTSPEZIFISCHEN RESISTENZ DURCH HEMMUNG DES EIWEISSSTOFFWECHSELS

Es wurde nachgewiesen, daß bei Ratten und Meerschweinchen durch Hemmung des Eiweißstoffwechsels mit Äthionin ein tödliches Krankheitsbild auch mit solchen stenoxischen Krankheitserregern hervorgerufen werden kann, welche sonst für Ratten und Meerschweinchen vollständig indifferent sind. Auf diese Weise gelang es, bei Ratten mit den Erregern der Teschener Krankheit (Schweinelähmung), mit dem Erreger der Virus-Hepatitis der Enten sowie mit dem der Hepatitis epidemica des Menschen eine letal ausgehende Erkrankung hervorzurufen. Von den an Teschener Krankheit eingegangenen Ratten konnte das zur Infektion benutzte Virus in der Schweinenierenepithel-Gewebskultur in allen Fällen zurückgewonnen werden. Die Virus-Hepatitis der Enten hat bei 40% der infizierten Ratten charakteristische, auch histologisch nachweisbare pathologisch-anatomische Veränderungen (Blutungen in der Leber, Gelbsucht) zustande gebracht. In der Leber der eingegangenen Ratten konnte das Virus mittels Hühnerembryo-Impfung nachgewiesen werden. Bei 50 bzw. 40% der Ratten und Meerschweinchen, die mit dem Stuhlfiltrat der an Hepatitis epidemica erkrankten Menschen infiziert wurden, entwickelte sich ein typisches pathologisch-anatomisches Bild (Gelbsucht). In der Leber der verendeten oder getöteten Tiere entstanden ähnliche Gewebsveränderungen, wie sie bei Hepatitis epidemica des Menschen wahrgenommen werden können. Mit dem Leberhomogenat der verendeten Tiere konnte das Agens auf die mit Äthionin vorbehandelten Tiere übertragen werden.

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UNTERSUCHUNG DER ANTIKÖRPERPRODUKTION VON KALTBLÜTERN

Wie Verff. festgestellt hatten, können Frösche bei 20—30° C gut immunisiert werden, und sie bewahren ihre Immunität, solange günstige Temperaturbedingungen vorliegen. Bei den ähnlich vorbehandelten, aber bei niedrigerer Temperatur (8° C) gehaltenen Kaltblütern können kreisende Antikörper erst dann nachgewiesen werden, wenn die Tiere nach der Immunisierung in eine Temperatur von 25—30° C verbracht werden. Die Antikörperproduktion findet also bei den Kaltblütern sowohl bei der Temperatur von 8° C als auch der von 20—30° C statt. Wahrscheinlich hängt es von der Temperatur ab, ob die Antikörper in den Kreislauf gelangen.

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UNTERSUCHUNG EINES NEUEN ANTITUBERKULÖSEN VAKZINE-STAMMES

Die biologischen, pathogenen und immunogenen Eigenschaften des attenuierten *Mycobacterium tuberculosis*-Stammes Nr. 115 wurden untersucht. Es gelang, vom Stamm eine isoniazidresistente Mutante zu gewinnen, deren immunogene Eigenschaften nicht geschwächt sind. Die pathogenen Eigenschaften des Stammes wurden an Meerschweinchen in neueren Versuchen untersucht und mit denen des BCG-Stammes verglichen. Die immunogenen Eigenschaften des Stammes sind besser als die des BCG-Stammes, was auch neuere Meerschweinchenversuche bestätig ten.

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VERSUCHE ZUR HERSTELLUNG EINER VAKZINE GEGEN DEN CHRONISCHEN PARATYPHUS DER SCHWEINE

Mit dem an Aluminiumhydroxyd-Gel adsorbierten, mit Formalin abgetötete *Salmonella typhi suis*-Bakterien enthaltenden Formol-Vakzine-Präzipitat konnten in der Praxis in den mit chronischem Schweine-Paratyphus (Voldagsen-Paratyphus) infizierten Wirtschaften folgende Resultate erzielt werden:

Von den nach der Ablaktation und im Frischlingsalter in der überwiegenden Mehrzahl der Fälle mit 1×4 ml Vakzine geimpften 1250 Schweinen sind 114 (9,12%), von den 1385 ungeimpften Kontrolltieren 246 (17,75%) an Vol-

dagsen-Paratyphus eingegangen. Daraus folgt, daß die Vakzine, 48,6% derjenigen ablaktierten Ferkel und Frischlinge vor dem Verenden bewahrte, die ohne Impfung an Voldagsen-Paratyphus eingegangen wären.

Die im Saugalter angewendete aktive Immunisierung erwies sich als viel wirksamer. Nach Impfung der Hälfte der Würfe in Abständen von 2—4 Wochen mit 2×2 ml Vakzine sind 7 der geimpften 1407 Ferkel (0,5%), bei der anderen Hälfte der Würfe dagegen 52 der zu Kontrollzwecken nicht geimpften 1445 Ferkel (3,6%) an Voldagsen-Paratyphus eingegangen.

Bei den Säuen im 3—4. Trächtigkeitsmonat löste 4—10 ml Vakzine keine Impfreaktion aus. Von den 129 Ferkeln der im Trächtigkeitsalter geimpften 12 Säue ist 1, von den 140 Ferkeln der 12 ungeimpften Kontrollsäue sind 15 an Voldagsen-Paratyphus eingegangen.

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UNTERSUCHUNG DER ANTIBAKTERIELLEN STAPHYLOKOKKENIMMUNITÄT

Die serologische Typenbestimmung bot die Möglichkeit, die Frage der antibakteriellen Staphylokokkenimmunität zu untersuchen. Die bisherigen Untersuchungen an Mäusen ergaben, daß die Staphylokokkenstämme eine ausgeprägte antibakterielle Immunität zustandebringen. Diese Immunität ist nicht typusspezifisch, da die serologisch abweichenden Stämme fast den gleichen Immunitätsgrad herbeiführen. Dieses Schutzantigen ist auch in den nach Boivin, Westphal und Morse hergestellten Extrakten anzutreffen. Aus den Untersuchungen ging fernerhin hervor, daß die Immunantwort vom optimalen Antigenstimulus abhängt. Sowohl in der Zone von sehr wenig, als auch von sehr viel Antigen ist der Schutz geringeren Grades als unter den mit der optimalen Antigenmenge immunisierten Mäusen.

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UNTERSUCHUNG DER ANTIGENEIGENSCHAFTEN UND CHEMISCHEN STRUKTUR VON BESTRAHLTEM EIWEISS

Die Antigenität und SH-Gruppen von Ovalbuminlösungen verschiedener Konzentrationen wurden vor und nach Röntgenbestrahlung untersucht. Im Rahmen des angewendeten Dosisbereichs konnten qualitative Antigenitätsveränderungen nicht festgestellt werden. Die ermittelten quantitativen Unterschiede werden — im Einklang mit den SH-Gruppenzahlbestimmungen — auf die Änderung im Zahlenverhältnis der SH-determinanten Gruppe zurück-

geführt. Aus den Ergebnissen wird die Schlußfolgerung gezogen, daß bei einer Strahlenschädigung der Eiweißstoffe die SH-Gruppen sowohl in biologischer, als auch in strahlenchemischer Beziehung am empfindlichsten reagieren.

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ÜBER DIE ZUSAMMENHÄNGE ZWISCHEN DEM DIFFERENZIALSPEKTRUM UND DER IMMUNOLOGISCHEN FUNKTION VON KRYOGLOBULINEN

Nach neueren Literaturangaben eignet sich die Differenzialspektralanalyse der Eiweißstoffe zur Registrierung der Molekülkonfiguration und inneren Strukturveränderungen.

Verff. studierten das Differenzialspektrum, die immunelektrophoretischen und Sedimentationseigenschaften sowie das mit F II sensibilisierte Latex-Agglutinationsvermögen von 10 verschiedenen Kryoglobulinen.

Unter den Makrokryoglobulinen zeigt in Säure das Differenzialspektrum derjenigen, die über Latex-Agglutinationsvermögen verfügen, Veränderungen entgegengesetzten Charakters als die Makrokryoglobuline, die zur Latex-Agglutination nicht imstande sind. Das Differenzialspektrum des normalen Gammaglobulins und des allein aus S-Komponenten bestehenden Kryoglobulins ändert sich in Säure auf gleiche Weise.

Von Karbamid wird das Differenzialspektrum der Kryoglobuline bedeutend, aber verschiedenartig verändert.

Die Untersuchungsergebnisse gestatten den Schluss, daß ein Zusammenhang zwischen der Variabilität der sekundären, tertiären Struktur der Kryoglobuline und der Molekülgröße sowie dem Latex-Agglutinationsvermögen vorausgesetzt werden muß.

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CHEMISCHE ANALYSE UND IMMUNBIOLOGISCHE UNTERSUCHUNG VON SALMONELLA TYPHI-ANTIGENEN

Anläßlich der Untersuchung der nach verschiedenen Verfahren gewonnenen *Salmonella typhi*-Antigene wurde zuerst die chemische Analyse der mit dem Trichloressigsäure-Verfahren auf zweierlei Weise (nach der Methode von Rauss bzw. Boivin) hergestellten Antigene durchgeführt.

In erster Linie ist ein Unterschied in der quantitativen chemischen Zusammensetzung der einen annähernd identischen Immunogenwert, aber abweichende Toxizität aufweisenden Antigene festzustellen. Die auf Poly-

saccharid bezogene Reinheit des Antigens mit niedrigerer Toxizität ist wesentlich (etwa 60—70mal) größer als die des 5mal toxischeren.

Bei wiederholten Untersuchungen konnte im Stamm *Salmonella typhi 2* neben den Hexosen (Galaktose, Glukose, Mannose) und 3-6-Didesoxyhexose (Tyvelose) auch eine Pentose (Xylose) nachgewiesen werden.

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NEUE METHODE ZUM PHOTOGRAPHIEREN DER BEI DER AGARDIFFUSIONSPROBE ENTSTEHENDEN PRÄZIPITATIONSLINIEN

Zwecks Vermeidung der sich bei der Herstellung qualitativ einwandfreier Negativbilder ergebenden Schwierigkeiten registriert Verf. die in der Agarschicht entstehenden Präzipitationslinien, indem er in Analogie zu dem beim Photographieren bekannten Kontaktkopieren von der die Präzipitationsstreifen enthaltenden Agarplatte als Negativ direkt ein Positivbild herstellt. Die Einzelheiten des Kontaktkopierens der Präzipitationslinien sowie die bei Anwendung dieser Methode gewonnenen Erfahrungen werden mit Hilfe von Photos demonstriert.

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UNTERSUCHUNG DER AVIDITÄT VON IMMUNSERA

Bei der gleichzeitig vorgenommenen Hämagglutinationsuntersuchung und biologischen Titration von Diphtherie- und Tetanus-Immunsera konnte festgestellt werden, daß eine enge und eindeutige Korrelation zwischen den Ergebnissen der beiden Methoden vorliegt: mit einem hohen Hämagglutinationswert geht ein hoher biologischer Wert einher und umgekehrt.

Bei einem Teil der Sera kamen jedoch 4—5fache Unterschiede zwischen den Resultaten der beiden Titrationen vor. Diese Beobachtung führten Verff. zum Studium der Aviditätsfragen.

Der Diphtherie-Antitoxin-Titer wurde auf dem Niveau LR/30 und LR/3000 nach der ic. Kaninchenmethode in 47 Kinder-, 50 Meerschweinchen-Sera und 22 hyperimmunen Pferdesera, weiterhin auf dem Niveau L+/10 und L+/400 an weißen Mäusen der Tetanus-Antitoxingehalt von 154 Kinder-, 163 Meerschweinchen- und 24 Pferdesera untersucht. Wie die Versuche ergaben, wird der aviditätsbedingte Wertunterschied vom Immunisierungsverfahren, von der Qualität des zur Titration benutzten Toxins und vom Ursprung der Sera in hohem Maße beeinflußt.

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HAUTREAKTIONEN AN DIABETISCHEN KANINCHEN

Verff. hatten nachgewiesen, daß der Jancsó-Test und das Shwartzmansche Phänomen bei Diabetes in hohem Maße gehemmt werden. Es wurde nun untersucht wie die mit Diphtherie-Toxin, mit dem Antigen-Antikörperkomplex zustandegebrachte Hauterscheinung und das umgekehrt passive Arthus-Phänomen bei diabetischen Kaninchen verlaufen.

Diabetes wurde durch iv. Verabreichung von 150 mg/kg Alloxan hervorgerufen. Die Titration des Diphtherie-Toxins nach Jensen ergab, daß die Hautreaktion bei den diabetischen Tieren mit schwächerer Intensität zutage trat als bei den normoglykämischen Kontrolltieren.

Die Herstellung des Antigen-Antikörperkomplexes erfolgte, indem man am Äquivalenzpunkt das Ovalbumin gegenüber erzeugte Kaninchen-Antiserum mit Ovalbumin präzipitierte und dann das Präzipitat in der 3—4fachen Menge des benutzten Antigens auflöste. Dieser Immunkomplex kam intrakutan zur Anwendung. Nach den Ergebnissen trat die auf diese Weise herbeigeführte Hautreaktion bei den diabetischen Kaninchen bedeutend schwächer in Erscheinung oder blieb ganz aus.

Auf Grund der bisherigen Versuchsresultate hat es den Anschein, daß auch das umgekehrt passive Arthus-Phänomen bei den diabetischen Tieren ein geringeres Ausmaß zeigt als bei den normoglykämischen Kontrolltieren.

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DIE QUANTITATIVEN HISTAMIN- UND SEROTONINVERHÄLTNISSE BEI ANAPHYLAXIE

An mit Pferdeserum sensibilisierten Meerschweinchen, Ratten und Hunden bestimmten Verff. nach anaphylaktischem Schock die Histamin- und Serotoninmenge im Blut sowie in der Leber und Lunge. Die gewonnenen Werte wurden mit dem Histamin- und Serotoningehalt in den Geweben der Kontrolltiere verglichen.

Nach dem anaphylaktischen Schock der Meerschweinchen steigt die Serotoninmenge in Blut und Leber in geringerem, in der Lunge in bedeutendem Maße. Der Leberhistamingehalt sinkt, der Histamingehalt im Blut steigt kaum, in der Lunge jedoch kräftig. Die Histamin- und Serotoninerhöhung in der Lunge erklärt ausreichend den dyspnoeartigen anaphylaktischen Schock. Bei der Anaphylaxie von Ratten nimmt der Serotonin- und Histamingehalt in der Leber signifikant ab, im Blut und in der Lunge jedoch zu.

Bei der Anaphylaxie von Hunden bleibt der Serotoningehalt in der Leber unverändert, während der Serotoningehalt im Blut prägnant, in der Lunge jedoch in geringerem Maße steigt. Die Serotoninzunahme im Blut stammt wahrscheinlich von den Mastzellen der Haut.

I. Jókay, A. Kiss, L. Kassai

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DIE WIRKUNG VON THIOL-VERBINDUNGEN AUF EINIGE ENDOTOXINREAKTIONEN

Es wurden die Wirkungen von Cystein und einzelnen SH-Verbindungen auf die von *E. coli*-Endotoxin in vitro ausgelöste Thrombozyten-Agglutination sowie Histamin- und Serotoninfreisetzung untersucht. Von Cystein in Konzentrationen über 5 *mM* wird die Thrombozyten-Agglutination sowie die Histamin- und Serotoninfreisetzung gehemmt. Reduziertes Glutathion, Mercaptoäthylguanidin und Thioglykolsäure wirken ähnlich, während Penicillamin keinen derartigen Effekt zeigt. Bei den für die lokale Shwartzmansche Reaktion vorbehandelten Kaninchen wird von den zur Auslösung iv. verabreichten 200—400 mg/kg Cystein die Leukozytenzahlsenkung im peripheren Blut nicht beeinflußt, aber die unter Endotoxinwirkung eintretende Thrombozytenzahlsenkung gehemmt. Ungeachtet dessen tritt bei den cysteinbehandelten Kaninchen das quantitative Shwartzman-Phänomen zeitlich und im Effekt ausgeprägt stärker zutage, als bei den Kontrolltieren. Diese Cysteinwirkung wird auf die Aktivierung von SH-Proteasen zurückgeführt.

BAKTERIENGENETIK

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DIE AUXOTROPHE DES BACILLUS ANTHRACIS

Die erfolgreiche Isolierung der Auxotrophe des *B. anthracis* und die Eigenschaften dieser Mutanten werden besprochen. Die Isolierung erfolgte nach zwei Verfahren: 1. Züchtung von UV-bestrahlten vegetativen Formen in Anwesenheit von 8-Azaguanin. 2. Die vegetativen Formen wurden der Wirkung von Äthylmethansulfonat ausgesetzt, wonach man die Bakterien Sporen bilden ließ. Die Sporen der den mutagenen Wirkungen ausgesetzten Bakterien wurden auf Hefe-Pepton-Nährboden ausgebreitet und die entwickelten

Kolonien auf einen Kaseinhydrolysat-Minimalnährboden repliziert. Auf diese Weise konnten Vitamine oder Nukleinsäure-Basen beanspruchende Auxotrophe gewonnen werden.

Nach dem 1. Verfahren wurden 2 auxotrophe Stämme unter 5763 Kolonien festgestellt. Bei Anwendung des 2. Verfahrens wurden in mehreren gesonderten Versuchen 9249 Kolonien auf ihren minimalen Nährstoffbedarf untersucht. Von diesen erwiesen sich 189 als auxotroph. Die Mehrzahl der Auxotrophen zeigte Purinbedarf; der Block entstand jedoch an verschiedenen Stellen, entweder in der Synthese von Pyrimidin oder bereits in der Synthese des Imidazol-Ringes. Außerdem waren in beträchtlicher Zahl auch Stämme vorhanden, die einen Defekt in der Pyrimidin-Base-Synthese aufwiesen. Unter den Stämmen mit Vitaminbedarf kamen häufig solche mit Biotin-Bedarf vor.

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WEITERE UNTERSUCHUNG VON TRANSDUZIERENDEN SUBTILIS-PHAGEN

In ihrer Antigenstruktur waren die von Verff. beschriebenen transduzierenden Phagen einheitlich und stimmten mit dem Takahashi-Stamm überein. Ein Unterschied lag jedoch gegenüber dem Thorne-Phag nicht nur in der Antigenstruktur, sondern auch in bezug auf den »host-range« sowie auf die Häufigkeit der gekoppelten Transduktion vor. Unter den transduzierten Subtilis-Phagen können somit Unterschiede vorkommen. Es scheint, daß nach dem im Laboratorium ausgearbeiteten temperierten Phag-Isolierungsverfahren in der Regel Phage mit identischen Eigenschaften gewonnen werden.

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DIE FOLGEN DER IN DER RIBONUKLEINSÄURESYNTHESE REGULATION NACHWEISBAREN STÖRUNG FÜR DIE PHYSIOLOGIE VON E. COLI

Bei den meisten *E. coli*-Stämmen steht die Regulation der Ribonukleinsäuresynthese unter strenger Aminosäurekontrolle. Als Resultat der im kontrollierenden Gen (RC-Locus) eingetretenen Mutation findet aber die RNS-Synthese auch in Ermanglung von Aminosäuren weiter statt (Relaxation). Werden diese Stämme im kompletten oder minimalen Nährboden gezüchtet, so wachsen und vermehren sie sich ebenso wie die normalen Stämme.

Überträgt man aber die RC-Mutanten vom kompletten in den minimalen Nährboden, so verlängert sich außerordentlich die Latenzzeit ihrer Vermehrung,

ja ein sehr beträchtlicher Prozentsatz der Bakterien dieser Kultur ist zur Adaptation überhaupt nicht imstande.

Das Fehlen des Adaptationsvermögens hängt damit zusammen, daß diese Bakterien je nach ihrem physiologischen Zustand auch in bezug auf die Regulation des die Aminosäuren synthetisierenden Systems an mehr oder weniger ausgeprägten Anomalien leiden. Sie können Leucin, Isoleucin, Serin, Cystein und Phenylalanin gegenüber hypersensibel sein.

Die Leucin-, Isoleucin-, Cystein- und Phenylalanin-Empfindlichkeit ist wahrscheinlich auf eine einheitliche Ursache zurückzuführen und als Spezialfall der Inhibition des Endproduktes aufzufassen. Der genauere Mechanismus der Serin-Empfindlichkeit ist einstweilen unbekannt. Dessen ungeachtet kann von Serin nachgewiesen werden, daß es eine spezifische Rolle in der Regulation der Ribonukleinsäuresynthese spielt.

Auf Grund dieser Versuchsergebnisse kann die im vorigen Jahr vertretene Auffassung, wonach die von Aminosäuren gesteuerte Regulation der RNS-Synthese mit Hilfe der Repressor-Theorie zu erklären sei, nicht länger aufrechterhalten werden, vielmehr wird die Aufstellung einer neuen Hypothese notwendig.

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ANOMALIEN IN DER VERTEILUNG EINIGER AUS DER KONJUGATION SERIN UND GLYCIN BEANSPRUCHENDER BAKTERIEN STAMMENDER REKOMBINATIONSTYPEN

Bei den Kreuzungen Hfr × F^- sind, wenn es sich bei F^- um ein Serin-Glycin beanspruchendes Bakterium handelte und die Rekombinanten mit Maltose, Xylose, Laktose oder Galaktose selektiert wurden, sämtliche auf diese Weise entstandenen Rekombinanten auch serin-glycin-prototroph geworden. Die Erscheinung läßt sich keinesfalls darauf zurückzuführen, daß das Serin-Glycin-Synthetisierungsvermögen an diese Markern gebunden sei. Die bisherigen Versuchsergebnisse sprechen eher für die Hypothese, daß man bei den erwähnten Selektionen einen großen Teil der entstandenen Rekombinanten verliert und nur die serin-glycin-prototroph gewordenen Rekombinanten Kolonien zu bilden vermögen. Die Erscheinung dürfte somit nicht einen genetischen, sondern einen physiologischen Hintergrund haben.

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DIE IMMUNOLOGISCHEN, PATHOGENEN UND BIOCHEMISCHEN EIGENSCHAFTEN VON MYCOBACTERIUM KANSASII UND SEINE REVERSION ZU MYCOBACTERIUM TUBERCULOSIS

Die Untersuchung der biochemischen, pathogenen, serologischen und immunogenen Eigensachaften von zwei *M. kansasii*-Stämmen hat ergeben, daß diese mit den Eigenschaften der *M. tuberculosis*-Stämme von attenuierter Virulenz weitgehend übereinstimmen.

Von beiden Stämmen wurde eine, infolge Dissoziation nicht pigmentbildende R-Variante isoliert und eine davon eingehend untersucht. Der untersuchte Stamm 232 R hat sich auch in seinen kulturellen und immunogenen Eigenschaften als attenuierter Tuberkelbazillus erwiesen.

M. kansasii stammt von einer im Menschen auftretenden Mutation des Tuberkelbazillus und kann als selbständige pathogene Bakterienart differenziert werden, doch deutet auf seinen Ursprung auch der Umstand hin, daß es in den typischen Tuberkelbazillus revertiert.

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ÜBER DIE GENETISCHEN EIGENSCHAFTEN DER POLIOVIREN

Aus der überimpfbaren Affennierenzellkultur (MK_3) wurde eine, attenuiertes Poliovirus 3 tragende Zellinie gebildet. Vier Monate danach wurde das Virus reisoliert und zwei seiner genetischen Merkmale (rct/40, d) mit denselben Merkmalen des Virus-Originalstammes verglichen. Es stellte sich heraus, daß der reisolierte Sabinsche Virusstamm (3. Typ), der sich ursprünglich bei 40° C nicht vermehrte ($rct/40^-$), nach 4 Monate langem Virustragen bei 40° C besser vermehrte als der wilde Poliovirus-Kontrollstamm $rct/40^+$. Außerdem änderte sich auch der d-Charakter des reisolierten Virusstammes.

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TRANSFORMATION DER STREPTOMYCIN-INDEPENDENZ MIT DER DNS VON RHIZOBIUM LUPINI ROUGH-STÄMMEN

Verf. stellte 1960 fest, daß die Streptomycinresistenz gesondert, aber auch gemeinsam transformiert wird; auch im Falle intraspezifischer Transformation ist die Transformationshäufigkeit sehr niedrig: 10^{-6}—10^{-7}. Ein und das selbe DNS-Präparat ist imstande, sowohl die »instabile«, als auch die »stabile«

Dependenz zu transformieren. Verf. untersuchte den Grad der durch die Transformation übertragenen Streptomycin-Dependenz bzw. -Resistenz und den Zusammenhang des transformierten Charakters mit der Größe der Kolonien.

Im Vortrag wird die Transformation der Streptomycin-Independenz besprochen. Nach Veränderung der Transformationsrichtung (reziproke Transformation) betrug die Transformationshäufigkeit $1 \times 10^{-4} - 3 \times 10^{-2}$.

Die Häufigkeit der mit der DNS von streptomycin-empfindlichen Donatoren, solchen mit niedriger und hoher Streptomycinresistenz und schließlich der streptomycin-independenten Donatoren (back-Mutante !) durchgeführten Transformationen wurde verglichen (Rezipient: streptomycin-dependenter Stamm), ebenso der Charakter der bei den verschiedenen Transformationen gewonnenen Transformanten (Streptomycin-Empfindlichkeit oder -Resistenz) bzw. der Grad der Streptomycinresistenz der streptomycin-independenten Transformanten.

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DIE STRAHLENEMPFINDLICHKEIT VON ESCHERICHIA COLI-KULTUREN

I. PHYSIOLOGISCHE FAKTOREN VON EINFLUSS AUF DIE STRAHLENEMPFINDLICHKEIT

Von

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(Eingegangen am 2. Mai 1963)

Zusammenfassung. Die Veränderungen in der Strahlenempfindlichkeit von *E. coli* B-Kulturen im Verlauf des Wachstumszyklus wurden untersucht. Es konnte festgestellt werden, daß sich die Strahlenempfindlichkeit während der Züchtung ändert. Die größte Veränderung entfällt auf die als kinetisch konstant bekannte Log-Phase. Es wurde ein Zusammenhang zwischen der Veränderung der Strahlenempfindlichkeit und des Elektrodenpotentials in der Kultur nachgewiesen. Auf dieser Grundlage kann aus der Elektrodenpotentialkurve auf den am meisten strahlenempfindlichen Zustand der Kultur geschlossen werden. Aus den Ergebnissen wurden theoretische und praktische Folgerungen gezogen.

Die Populationen »freier Zellen« (z. B. Mikroorganismen) kommen in der Erforschung radiobiologischer Mechanismen sehr ausgedehnt zur Anwendung. Durch ihre Benutzung werden viele Faktoren ausgeschlossen, die bei den Versuchen mit organisierten Geweben nicht außer acht gelassen werden dürfen. Ein wesentlicher Gesichtspunkt ist fernerhin, daß die Bewertung des letalen Effektes der Strahlung eine verhältnismäßig einfache Aufgabe darstellt. Unter dem Begriff der letalen Strahlenwirkung [1] verstehen wir den Zustand, in dem eine »freie Zelle« ihr Reproduktionsvermögen verloren hat. Zugleich können viele andere ihrer meßbaren Funktionen — die Aktivität einzelner Enzyme [2], die adaptive Enzymsynthese [3] und Atmung [4] — unberührt bleiben.

In den letzten Jahren hat man vielfach die Bedingungen untersucht, die die Strahlenempfindlichkeit der Zellen verändern können bzw. verändern. Diese Faktoren lassen sich in vier Gruppen [1] einordnen:

(I) Physiologische und morphologische Faktoren.

(II) Physikalische Verhältnisse im Augenblick der Bestrahlung.

(III) Modifizierende Stoffe, die während der Bestrahlung anwesend sein müssen.

(IV) Postirradiative Züchtungsverhältnisse.

Im Vordergrund unseres Interesses stehen diejenigen chemischen Substanzen, die die Strahlenempfindlichkeit der Zellen teils im Augenblick der Bestrahlung, teils als nach der Bestrahlung wirkende Faktoren herabsetzen.

Vor dem Beginn derartiger Versuche wünschten wir zu klären, welche Rolle den physiologischen Faktoren in der Entwicklung der Strahlenempfindlichkeit zukommt.

Aus den Untersuchungen von HOUTERMANS [5] wissen wir, daß nach Rtg-Bestrahlung eines *E. coli* HIG-Stammes die Kurvenformen der Überlebenden vom Alter der Kultur abhängig waren. Im Falle »junger« Kulturen hatte die Kurve Sigmoidform, im Falle »alter« Kulturen war sie von exponentialer Form. (Theoretisch unterscheidet man 3 überlebende Kurventypen: Typ A = exponential; Typ B = annähernd exponential mit resistentem Ende; Typ C = sigmoidförmig [6].)

STAPLETON [7] untersuchte die Veränderungen der Strahlenempfindlichkeit des *E. coli* B/r-Stammes im Verlauf eines Züchtungszyklus. In den 6 Stunden der Log-Phase erfolgten die meisten Veränderungen, als sich die Bakterien mit annähernd gleicher Geschwindigkeit vermehrten.

Demgegenüber fand MARKOVICH [1] bei der Untersuchung der Log-Phase von *E. coli* B/r- und K_{12}(S)-Kulturen keine Veränderung der Strahlenempfindlichkeit in der Log-Phase.

Gestützt auf diese Angaben nahmen wir unsere Versuche mit *E. coli* B-Kulturen vor, wobei wir die letale Wirkung der ionisierenden Strahlung untersuchten, die auch bei unseren Versuchen zum Verlust des Reproduktionsvermögens führte.

Material und Methoden

In Nährböden ließen wir die mit gleicher Zellzahl geimpften Kulturen bei 37° C wachsen. Von den stündlich entnommenen Proben wurden die absolute und lebende Zellzahl sowie die Trockensubstanz bestimmt und auf Grund dieser Werte Strahlenempfindlichkeits- und Wachstumskurven verfertigt.

Die Dosiswirkungskurve wurde an Hand von Zellen bestimmt, die aus 14stündigen Kulturen stammten. Die Bestrahlung nahmen wir in physiologischer NaCl-Lösung bei gleicher Zellzahl vor. Hiernach wurden die Zellen auf Nähragar ausgebreitet, bei 37° C 24^h inkubiert und die Klone gezählt.

Die Bestrahlung erfolgte mit der Siemens-Stabilivolt-Apparatur (bei 180 kV und 12 mA) ohne Filter. Die Fokus-Objektiventfernung betrug 6 cm, die Dosisleistung 800 *r*/min.

Bei der Aufnahme der Dosiswirkungskurve wechselten die Dosen zwischen 800 und 6000 *r*.

Die Veränderungen in der Strahlenempfindlichkeit der Kulturen untersuchten wir bei konstanter Dosis (1600 *r*). Als Maßstab der Strahlenempfindlichkeit diente das bei der Standarddosis ermittelte prozentuale Überleben.

Das Elektrodenpotential der Kulturen wurde mit dem pH-Meter gemessen bzw. mit dem Punktographen registriert.

Ergebnisse

Die von den aus der 14stündigen Kultur gewonnenen Zellen aufgenommene Dosiswirkungskurve zeigt Abb. 1. Die ermittelte Überlebenskurve gehört zum Typ B, d. h. sie hat ein resistentes Ende. Dies bedeutet, daß die Zellen der 14stündigen Kultur in bezug auf die Strahlenempfindlichkeit keine homo-

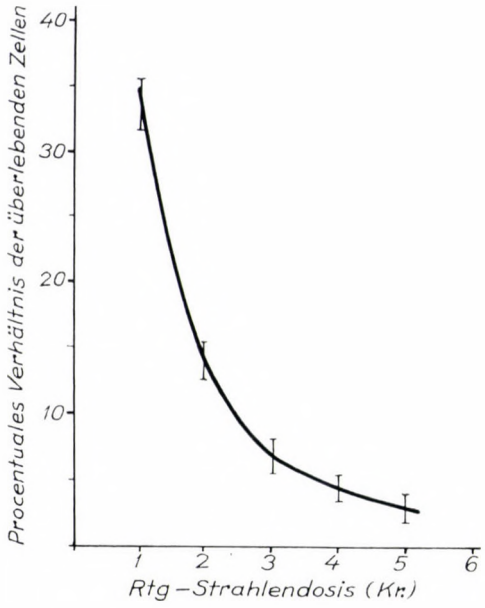

Abb. 1. Dosiswirkungskurve von den Zellen der 14stündigen Kultur des *E. coli* B-Stammes

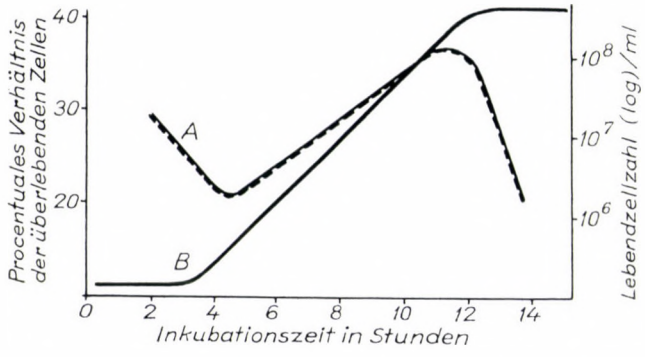

Abb. 2. Veränderungen in der Strahlenempfindlichkeit des *E. coli* B-Stammes im Verlauf der Züchtung. Kurve A = Entwicklung der Strahlenempfindlichkeit der Zellen unter Wirkung von 1600 *r*; Kurve B = Vermehrung der Bakterien

gene Population bilden, sondern etwa 10% der Zellen resistenter sind als die Mehrzahl der Population.

Abb. 2 stellt gleichzeitig die Wachstums- und Strahlenempfindlichkeitskurve der Kultur dar. Im Nährmedium folgt nach einer 2—3stündigen Latenz- und anschließenden 6—8stündigen exponentialen Wachstumsphase eine stationäre Phase. Die beiden letzten Phasen des vollständigen Züchtungszyklus haben wir vom Gesichtspunkt der Strahlenempfindlichkeit nicht mehr unter-

sucht. Die gestrichelte Linie zeigt auf Grund der stündlich entnommenen Proben die relative Strahlenempfindlichkeit der *E. coli*-Zellen an. Wie ersichtlich, ist die Strahlenempfindlichkeit der Kultur am Anfang der Log-Phase am größten, dann nimmt sie gleichmäßig ab und erreicht das Minimum am Ende der Log-Phase; in der stationären Phase wächst die Strahlenempfindlichkeit wieder.

Im weiteren befaßten wir uns mit der Frage, worauf die im Verlauf der Züchtung eintretenden Veränderungen der Strahlenempfindlichkeit beruhen. Wie aus Abb. 2 hervorgeht, konnte aus dem Ergebnis nicht der eindeutige Schluß gezogen werden, daß die Veränderungen in der Strahlenempfindlichkeit mit dem kinetischen Zustand der Zellvermehrung, d. h. mit der Lebendzellzahl der Kultur oder mit ihrem Alter zusammenhängen. Die Wachstumskurve und Strahlenempfindlichkeitskurve verlaufen ähnlich, sind aber keineswegs identisch. Wahrscheinlich müssen die Veränderungen in der Strahlenempfindlichkeit auf einen physiologischen Zustand der Kultur zurückgeführt werden, der sich auch in der kinetisch konstanten Kultur, in der Wachstumsphase oder Log-Phase, ändert.

Bei anderen Untersuchungen gelangten wir zu der Schlußfolgerung, daß der physiologische Zustand, die Vitalität der Kultur neben der Wachstumskurve auch mit ihrem Elektrodenpotential ausgedrückt werden kann. Deshalb demonstrieren wir in Abb. 3 sowohl die Elektrodenpotentialkurve als auch die Wachstums- und Strahlenempfindlichkeitskurve der Kultur. Wie deutlich zu sehen, läßt sich im Verlauf der Züchtung eine Parallele zwischen der Strahlenempfindlichkeits- und Elektrodenpotentialkurve ziehen.

Besprechung

Diese mit *E. coli* durchgeführten Versuche bestätigen eindeutig die Feststellung STAPLETONS, daß die größte Veränderung der Strahlenempfindlichkeit während des Wachstums der Kultur in der kinetisch als konstant bekannten Log-Phase erfolgt.

Bei unseren Versuchen fanden wir die Zellen am Ende der Lag-Phase bzw. zu Beginn der Log-Phase am empfindlichsten und am Ende der Log-Phase am resistentesten. Wir nehmen an, daß diese Veränderungen mit denjenigen physiologischen bzw. biochemischen Geschehnissen zusammenhängen, die im Verlauf der Züchtung in den Zellen der Kultur vor sich gehen. Am Ende der Latenzphase besteht maximale enzymatische Tätigkeit, dann bereiten sich die Zellen gleichsam auf die logarithmische Vermehrung vor (an der Nukleinsäuren- und Eiweißsynthese teilnehmende Enzyme, Atmungsfermente usw.).

Die Elektrodenpotentialkurve bildet unserer Meinung nach den Index dieser Enzymaktivität, und sie verläuft bei unseren Versuchen mit den Veränderungen in der Strahlenempfindlichkeit parallel.

Die obige Entwicklung der Strahlenempfindlichkeit von *E. coli* B bedeutet in praktischer Beziehung, daß die in der kinetisch als konstant betrachteten Log-Phase entnommene Zellprobe bei Strahlenempfindlichkeitsuntersuchungen die Reproduzierbarkeit des Versuches nicht gewährleistet. Bei strahlenbiologischen Versuchen benutzt man die Zellen der Kultur am zweckmäßigsten

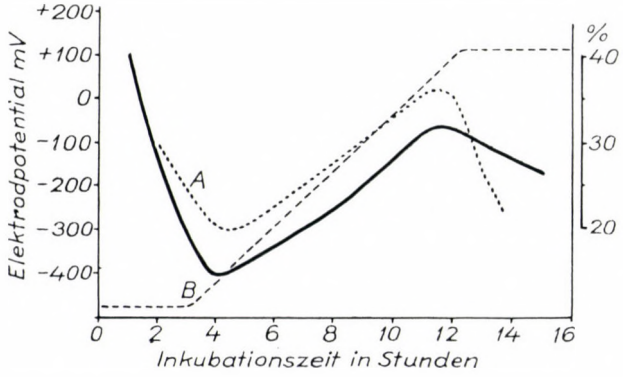

Abb. 3. Elektrodenpotentialkurve des Stammes *E. coli* B in der 14stündigen Inkubationszeit. A = Strahlenempfindlichkeitskurve; B = Wachstumskurve

im empfindlichsten Zustand. Dieser Zustand der Kultur wird beim Stamm *E. coli* B eindeutig vom Inflexionspunkt der Elektrodenpotentialkurve determiniert

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ADENOVIRUS HAEMAGGLUTINATION-INHIBITING ANTIBODIES IN HUMAN SERA

By

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Summary. Antibodies against two strains of type 8 and one strain each of types 9, 10, 11 and 16 of adenovirus have been examined by the haemagglutination-inhibition test in sera of persons convalescing from epidemic keratoconjunctivitis. Out of the total of 122 sera tested only 8 per cent had titres below 1 : 16 against type 8 virus. In a total of 42 serum pairs the acute samples were negative in 80 per cent while the convalescent ones in 5 per cent. A certain titre increase against types 9 and 10 while none against type 11 was demonstrable. Sera from 79 persons having had the disease in the past revealed the persistence of the haemagglutination inhibiting antibodies for a year. Triple serum samples were taken from a total of 23 persons. The examination of these sera had shown that the titres were higher in the 4th to 6th months of convalescence than those found one month after infection.

The incidence and titres of antibodies against types 8, 9, 10 and 13 of adenovirus were essentially the same in 350 sera from healthy persons. The average incidence of positivity was 42 to 47 per cent. A higher incidence was found in the 15 to 45 years age group than in the younger and older age groups. The incidence of antibodies against type 11 was low (average, 15 per cent) with a peak in the age group of 31 to 45 years. No haemagglutination-inhibiting antibody was found against type 16 adenovirus in sera of healthy persons or subjects convalescing from epidemic keratoconjunctivitis.

The haemagglutination inhibition test (HI) was first applied in adenovirus serology by ROSEN [1], who succeeded in demonstrating a type-specific titre increase in paired sera from persons infected with any of the types 1 to 7 of adenovirus [2]. The HI test was successfully applied by us for examining the strains isolated and for the study of paired sera from patients during an outbreak of epidemic keratoconjunctivitis in Budapest in 1961—62 [3]. The results obtained by the HI test were supported by neutralization and complement fixation (CF) tests. It was found that the HI was not less valuable than the neutralization test; moreover, the titres obtained by the HI test were generally superior to those yielded by the latter [4]. In addition, the HI test was found to be more advantageous than the CF test in several respects.

The present paper reports on some further studies on epidemic keratoconjunctivitis and on the serological examination of healthy subjects for antibodies against types 8, 9, 10, 11, 13 and 16 of adenovirus.

Materials and methods

Virus. The virus stocks used throughout the experiments were prepared in the usual manner in Detroit-6 and HeLa cell cultures [5]. The harvests were frozen and thawed repeatedly, then centrifuged and the supernatant kept at —20° C until used. Strains 9, 10, 11, 13

and 16 were kindly supplied by DR. VILČEK. The "Ijima" prototype 8 strain was obtained from DR. JAWETZ. The other type 8 strain was isolated in our laboratory from a case of epidemic keratoconjunctivitis during the 1961—62 epidemic in Budapest [3].

Sera : Serum samples were obtained from subjects who presented themselves with epidemic keratoconjunctivitis at different out-patient departments in Budapest. Normal sera were collected from healthy persons with no history of epidemic keratoconjunctivitis. The blood samples were centrifuged and the sera stored at −20° C. Before testing the sera were treated with bolus alba and absorbed with appropriate red blood cells (RBC) if needed.

RBC suspension. Washed rat, rhesus or human group "O" RBC suspensions of 1 per cent concentration in saline were used in the tests. The rat RBC suspensions were stabilized with 1 per cent normal rabbit serum [1, 6].

HI reaction. The reaction was performed in TAKÁTSY's Microtitrator apparatus [7] with 4 haemagglutinating (HA) units of virus, as described earlier [3].

Results

A total of 122 sera from patients convalescing from epidemic keratoconjunctivitis was studied for HI antibodies against the following six strains of adenovirus: type 8 strain isolated in our laboratory and prototype strains 8, 9, 10, 11 and 16. Results are presented in Table I. It can be seen that only 4 per cent of the sera gave negative results when tested against the strain isolated during the epidemic. Titres below 1 : 16 were observed in an additional 4 per cent of the total. All other sera gave higher titres. More than 90 per cent of the sera were positive also against prototype strains of type 8 and 9. The incidence of positivity was 68 and 32 per cent with strains 10 and 11, respectively.

Table I

Incidence of HI antibodies against 5 strains of adenovirus in sera of 122 persons convalescing from epidemic keratoconjunctivitis

HI titres (reciprocals)		Distribution of antibody levels against adenovirus types				
		8 (p)	8 (e)	9	10	11
<4	No	8	5	9	39	83
	%	7	4	7	32	68
4—8	No	9	5	19	32	34
	%	7	4	16	26	28
16—32	No	50	47	42	27	5
	%	41	39	34	22	4
64—128	No	42	50	43	23	—
	%	34	41	36	19	—
256—512	No	12	10	9	1	—
	%	10	8	7	1	—
1024	No	1	5	—	—	—
	%	1	4	—	—	—

Explanations:
(p) = prototype strain
(e) = strain from the actual epidemic

Type 16 was omitted from the Tables as neither the convalescent nor the healthy sera were found to contain HI antibodies against this type.

Paired sera obtained from a total of 42 patients with epidemic keratoconjunctivitis were tested for HI antibodies using the above mentioned six strains of adenovirus. Of these sera, 38 were studied also with other serological

Table II

Incidence of HI antibodies against 5 strains of adenovirus in paired sera of 42 patients with epidemic keratoconjunctivitis

HI titres (reciprocals)	Percentage distribution of antibodies against adenovirus types									
	8 (p)		8 (e)		9		10		11	
	A	C	A	C	A	C	A	C	A	C
<4	79	7	77	5	74	5	83	43	69	67
4—8	12	7	7	7	12	12	5	12	24	26
16—32	7	27	14	26	12	35	10	19	7	7
64—128	2	43	2	38	2	38	2	24	—	—
256—512	—	14	—	14	—	10	—	2	—	—
1024	—	2	—	10	—	—	—	—	—	—

Explanations:
A: Serum samples from acute stage of illness
C: Serum samples from convalescent stage of illness
(p) = prototype strain
(e) = strain from the actual epidemic

methods [3]. Table II presents the acute and convalescent HI titers of the 42 serum pairs. The 80 per cent incidence of negative HI in the acute sera with type 8 strains decreased to 5 to 7 per cent in the convalescent ones. The titres exhibited remarkable increases, except in two cases where the increase was only twofold. A similar increase was observable against type 9 strain. Titres against type 10 strain increased by 40 per cent while those against type 11 exhibited no change either in incidence or in value.

Triple serum samples were available from 23 patients. The first sample was taken at the onset of the acute phase, the second 3 to 4 weeks later and the third 4 to 6 months after onset of the disease. In most of the third samples a further increase of titre was observable againts type 8, 9 and 10 of adenovirus, as shown in Fig. 1.

In Table III are shown the results of an analysis of the data from Table I, according to the interval between the onset of disease and the sampling of blood. Out of the total of 122 sera tested, 79 were sampled after an interval of more than 3 months, 32 after 3 to 6 months, and 47 after 7 to 12 months. The average titres and the incidence of those above 1 : 16 have shown, that even after 12 months HI antibodies against the aetiological agent were still present

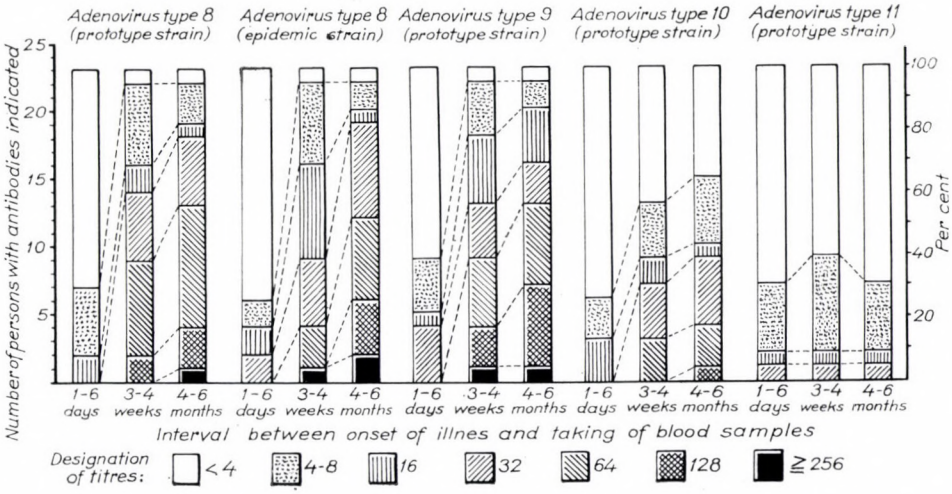

Fig. 1. Incidence of HI titres against 5 adenovirus strains in serum samples of 23 patients with epidemic keratoconjunctivitis at various points of time after onset of the disease

Table III

Average of HI titres and incidence of ≧ (1 : 16) *titre values against 5 adenovirus strains in 32 and 47 serum samples obtained 3—6 and 7—12 months, respectively, after onset of epidemic keratoconjunctivitis*

Adenovirus type	Average of HI titres in samples		Incidence of ≧ (1 : 16) titres (%) taken after	
	3—6 months	7—12 months	3—6 months	7—12 months
8 (p)	68	46	91	82
8 (e)	109	62	94	94
9	72	43	91	61
10	23	15	57	44
11	3	2	6	—

Explanations:
(p) = prototype strain
(e) = strain from the actual epidemic

at the same frequency and had the same titres as those in Table II. Similar results were obtained with type 9 of adenovirus, while with type 10 both the average titres and the incidence of those above 1 : 16 was lower.

Serum samples from 350 healthy persons were examined for HI antibodies against type 8, 9, 10, 11, 13 and 16 of adenovirus. The average titres and the incidence of the individual titre values are presented in Fig. 2. The incidence was similar for all strains but type 11. For the former negative

results were obtained in 53 to 58 per cent and titres lower than 1: 16 for an additional 26 to 29 per cent. These figures were remarkably higher for type 11 virus.

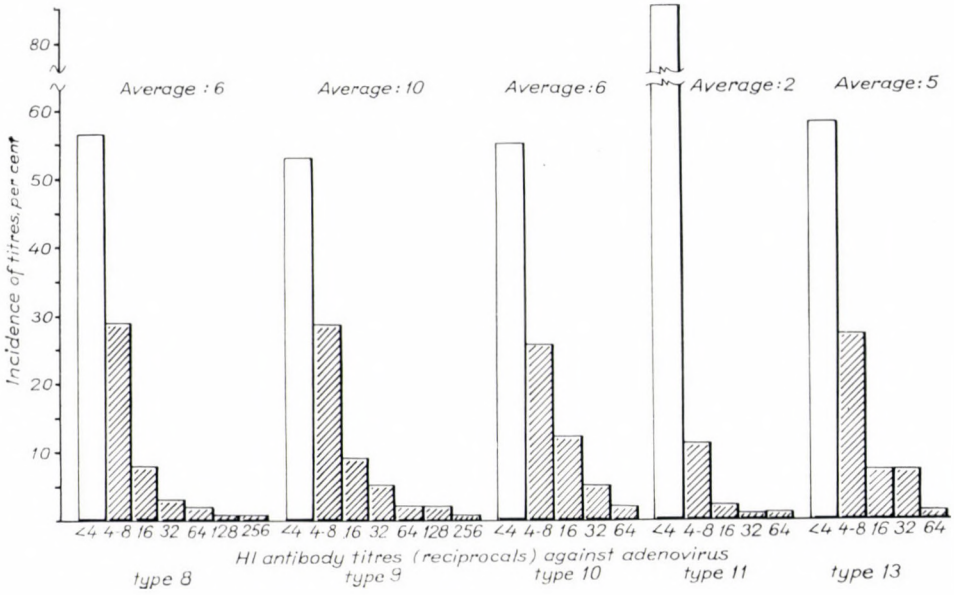

Fig. 2. Percentage distribution of HI antibody levels against 5 adenovirus strains in sera of 350 healthy persons

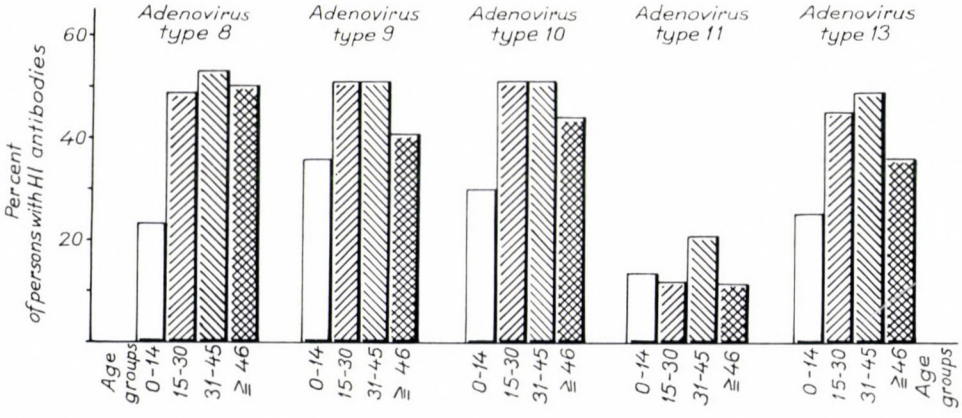

Fig. 3. Incidence of HI antibodies in sera of 350 healthy persons grouped according to age

The incidence of positive HI tests in 4 different age groups is presented in Fig. 3. The peak occurred in the age group of 15 to 45 years for all types save type 11; for this type a similar peak was observable in the age group of 31 to 45 years.

The numerical values of the positive titres for types 8, 9, 10 and 13 were practically identical, thus only those for type 10 were tabulated in detail in Table IV. The incidence of positive titres below 1 : 16 was about 52 to 60 per cent for the average and 70 per cent for the age groups below 14 years. Different results were obtained with type 11 strain, as shown in Table V. In this case the incidence of low titres among the scarce positive results was relatively high (average, 77 per cent).

Table IV

Incidence of HI antibodies against adenovirus type 10 in sera of 350 healthy persons grouped according to age

Age groups (years)	Number of sera tested	Positive sera		Percentage distribution of titres*			
		Number	%	4—8	16	32	64
0—14	78	24	31	71	17	8	4
15—30	149	75	51	58	25	9	8
31—45	61	31	51	52	35	13	—
≧46	62	26	42	54	31	11	4
Average			45	59	27	10	4

* Reciprocals

Table V

Incidence of HI antibodies against adenovirus type 11 in sera of 350 healthy persons grouped according to age

Age groups (years)	Number of sera tested	Positive sera		Percentage distribution of titres*			
		Number	%	4—8	16	32	64
0—14	78	11	14	82	18	—	—
15—30	149	20	13	60	20	15	5
31—45	61	13	21	77	15	—	8
≧46	62	8	13	87	13	—	—
Average			15	77	16	4	3

* Reciprocals

Discussion

JAWETZ *et al.* [8] were the first to identify type 8 of adenovirus as the aetiological agent of epidemic keratoconjunctivitis and to demonstrate the presence of type-specific antibodies in the sera of patients and convalescents

[8, 9]. This observation was later supported by other authors both in natural [10] and in artifical infections [11]. All these studies were, however, made with CF and neutralization technique while no data on similar studies with the HI test are available in the literature. In the course of the epidemic keratoconjunctivitis in Budapest we applied the HI test for aetiological studies and succeeded in revealing type 8 adenovirus as the pathogen [3]. This observation has reliably been supported by data obtained with 122 convalescent sera, as presented in Table I. A similar conclusion may be drawn from the examination of 42 paired sera tabulated in Table II. Thus the HI test appeared to be a valuable method for the laboratory diagnosis of epidemic keratoconjunctivitis just like for that of certain other adenovirus infections of the upper respiratory tract [2].

Results obtained with the other 3 types were in agreement with the well-known cross reaction between types 8 and 9 in both the neutralization [12] and the HI [1] tests. We are inclined to explain the increases in titre against type 10 also by the presence of a similar, though less marked cross reaction. This problem has been dealt with in a separate paper [13]. On the other hand, no significant changes were demonstrable in the HI titres of acute and convalescent sera when tested against type 11 of adenovirus.

The negative results obtained against type 16 were in agreement with the observations of ROSEN *et al.* [14] who failed to demonstrate the presence of HI antibodies against this type even in case of proven type 16 infections throughout a long term survey in a children's collective.

Since neutralizing antibodies are known to persist only for a limited time after the infection [9], it appeared to be of a certain interest to study the persistence of HI antibodies under similar conditions. Reports on a more prolonged persistence and higher titres attained by HI than by CF and neutralizing antibodies [3, 4] rendered such a study even more justified. Our results were in perfect agreement with the above observations. As shown in Table III, HI titres of 1 : 16 or higher against type 8 strains were observed in 94 per cent after 7 to 12 months following the infection. The average titres were significantly higher against all the types tested but type 11, as compared to those in sera of healthy persons (Fig. 1, 2).

The titre changes of triple serum samples presented in Fig. 1 suggested that in a number of cases the titres continued to increase after the first month following the disease and attained peak values only later. This seemed to be an adequate explanation of the fact that even after a year relatively high titres were observed.

Several authors have recently studied the incidence of CF and neutralizing antibodies against different types of adenovirus in sera of healthy persons. Positive reactions were observed in 30 to 50 per cent, with the highest ratio in children and young adults [15, 16, 17, 18, 19]. Our results are in agreement

with those cited [20]. Literary data on the types examined by us are, however, relatively scarce and even these have been obtained by the neutralization test.

JAWETZ *et al.* [21] and DREIZIN [22] found, in agreement with our results [23], an about 5 per cent, other authors [24] a 9 per cent incidence of antibodies against type 8 adenovirus. According to data from Taiwan, neutralizing antibodies against types 8 and 10 were found in more than 50 per cent of the sera of children; in adults the incidence was much lower [25]. In Italy during an epidemic keratoconjunctivitis survey low titre neutralizing antibodies were found in 8 out of 24 control sera against type 8 of adenovirus [10].

The incidence of HI antibodies against types 9, 10 and 13 in the sera of the 350 healthy persons examined by us (see Fig. 2) was in good agreement with that found by other authors. For type 11, however, the incidence was remarkably lower, 85 per cent of the sera being negative and an additional 11 per cent having titres below 1 : 16. This finding suggested that type 11 was less frequent in Hungary than the other three types tested. For type 8, however, the incidence was essentially the same as for types 9, 10 and 13.

The incidence of positive HI tests showed a maximum in the age group of 15 to 45 years, while for type 11 this maximum was between 31 to 45 years of age. Thus none of the types examined seemed to belong to those causing upper respiratory tract disease in children.

Our results obtained with type 8 differed somewhat from those known from the literature. The discrepancy may be explained as follows. (1) In our previous reports [13, 26] we have already pointed to the fact that the HI titres of sera were always higher than the neutralizing or CF titres. One may therefore suppose that sera with 1 : 16 or lower HI titres would yield a negative neutralization test, thus showing lower values for the incidence of positive sera. (2) Serum samples were collected within a few months following the epidemic in a department in Budapest, a town severely involved in the epidemic. This supposition is in agreement with certain results [27] showing that in sera taken before the epidemic from healthy persons the incidence of HI antibodies against type 8 was scarcely 5 per cent while this figure increased to 60 per cent in samples collected after an epidemic had taken place in the area. (3) The distribution of seropositivity according to age groups may be explained by the fact that the Hungarian epidemic had attacked mainly adults. A similar epidemiology was observed in the United States of America, while mainly children were involved in the epidemics observed in Taiwan and Japan.

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DIE WIRKUNG VON TUBERKULIN AUF DIE ATMUNG VON ASCITESZELLEN IN VERSCHIEDENEN ZEITPUNKTEN NACH INFEKTION MIT MYCOBACTERIUM TUBERCULOSIS

Von

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Zusammenfassung. Die Atmung der Asciteszellen von Meerschweinchen in Anwesenheit von Tuberkulin zu verschiedenen Zeitpunkten nach Infektion mit virulenten *M. tuberculosis* wurde an gesunden, mit BCG vakzinierten, vakzinierten und desensibilisierten, sowie mit INH und Streptomycin behandelten Tieren untersucht.

1. Die Atmung der Asciteszellen war nur 24 Stunden nach der Infektion in Anwesenheit von Tuberkulin erhöht; 3, 14 bzw. 42 Tage nach der Infektion war die Atmungserhöhung nicht mehr nachweisbar.

2. Die Atmung der Zellen der 6 Wochen vor der Infektion mit BCG immunisierten Tiere war in Anwesenheit von Tuberkulin 24 Stunden nach der Infektion gleichfalls erhöht; während aber die Wirkung bei den nicht immunisierten infizierten Tieren 3 Tage nach der Infektion nicht mehr nachgewiesen werden konnte, war sie bei den immunisierten Tieren auch nach 3 Tagen noch unverändert vorhanden.

3. Die Atmung der Asciteszellen der immunisierten, aber mit Tuberkulin desensibilisierten Meerschweinchen war in Anwesenheit von Tuberkulin 3 Tage nach der Infektion ebenso wie die der immunisierten, aber nicht sensibilisierten Tiere erhöht.

4. Die Atmung der Zellen von infizierten und mit INH behandelten Tieren war in Anwesenheit von Tuberkulin sowohl 24 Stunden als auch 3 Tage nach der Infektion gesteigert. Streptomycinbehandlung übte keine derartige Wirkung aus.

In den Zellen der mit Mykobakterien infizierten Tiere treten nach der Infektion verschiedene Veränderungen in Erscheinung. So stellten Dittmar und Sixel [2] fest, die Atmung und Glykolyse der Zellen von infizierten Tieren seien gesteigert. Einige Autoren wiesen nach, die erwähnten Veränderungen könnten schon vor Erscheinen der Hauttuberkulinempfindlichkeit bei den infizierten Tieren konstatiert werden [3, 4, 10, 17]. Bei den Untersuchungen der Zellen des peritonealen Exsudates von Meerschweinchen fanden auch wir, daß bereits 24 Stunden nach der Infektion — noch vor dem Auftreten der Hauttuberkulinempfindlichkeit — die Tuberkulinreaktion mit den Zellen passiv übertragen werden kann; in Anwesenheit von Tuberkulin nimmt die Atmung der Asciteszellen zu [14, 16].

In Fortsetzung dieser Versuche untersuchten wir im Rahmen der hier besprochenen Arbeit, wie sich die atmungsteigernde Tuberkulinwirkung zu verschiedenen Zeitpunkten nach der Infektion gestaltet. Die Untersuchungen nahmen wir an Asciteszellen von zuvor mit BCG immunisierten und nicht vakzinierten Meerschweinchen vor.

Material und Methoden

Verwendete Tiere. Für die Versuche benutzten wir aus eigener Zucht stammende, 200—300 g schwere Meerschweinchen beider Geschlechts.

Verwendete Mykobakterienstämme. Es wurden die 21 Tage alte Kultur des internationalen Stammes $H_{37}Rv$, die 14 Tage alte Kultur des Stammes Ravenel, die 21 Tage alte Kultur der Stämme BCG bzw. $H_{37}Ra$ sowie die 2 Tage alte Kultur von *M. friburgensis* benutzt. Die Stämme züchteten wir auf dem von SZABÓ modifizierten Sulaschen Nährboden. Bei den Untersuchungen kamen in physiologischer Kochsalzlösung suspendierte Bazillenmengen von 1 mg/ml bzw. 0,01 mg/ml Halbfeuchtgewicht zur Anwendung.

Tuberkulin. Wir benutzten das von der Impfstoffabrik Human (Budapest) erzeugte Alt-Tuberkulin sowie das von Statens Serum Institut (Kopenhagen) produzierte PPD.

Infektion der Tiere und Gewinnung der Zellen. Die Tiere wurden mit 1 mg/ml der erwähnten Stämme intraperitoneal infiziert. Zwecks Gewinnung der entsprechenden Zellmenge provozierten wir durch einmalige Zufuhr von 8 ml Paraffinöl 24 Stunden vor der Infektion bzw. durch Verabreichung von 5 ml 0,01 mg/ml Glykogen 5 Tage hindurch eine sterile Entzündung in der Bauchhöhle. In die Bauchhöhle der auf diese Weise behandelten und noch Mantoux-negativen Tiere spritzten wir zu verschiedenen Zeitpunkten nach der Infektion 20 ml heparinhaltige Kochsalzlösung ein, wonach die Tiere mit Chloroform getötet wurden. Nach Massieren öffneten wir die Bauchhöhle und saugten die Flüssigkeit mit der Pasteur-Pipette ab. Die Flüssigkeit wurde zentrifugiert, danach suspendierten wir die Zellen in Ringer-Lösung. Damit genügend Zellen für die Untersuchungen zur Verfügung stehen, bereiteten wir aus den vermischten Asciteszellen mehrerer gleichartig behandelter Tiere 9 ml Suspension.

Immunisierung der Tiere. Die Meerschweinchen wurden mit 1 mg/ml einwöchiger BCG-Vakzine subkutan geimpft. 6 Wochen nach der Impfung wurden die Tiere im Verhältnis 1 : 10 Mantoux-positiv. Nunmehr infizierten wir sie mit virulenten Bakterien.

Desensibilisierung der immunisierten Tiere. Bei den nach diesem Verfahren immunisierten Tieren bestimmten wir die Tuberkulin-Schwellenempfindlichkeit; die Desensibilisierung begannen wir mit derjenigen Verdünnung, bei der die Tuberkulinprobe noch negativ ausfiel. Von der Schwellenverdünnung ausgehend, wurde 0,1 ml zweitäglich subkutan in ansteigender Dosis verabreicht. Die 3 Tage nach der letzten Alt-Tuberkulin-Injektion ausgeführte Probe mit 1 : 10 verdünntem Tuberkulin (0,1 ml) erwies sich als negativ.

Isoniazid- und Streptomycinbehandlung. INH und Streptomycin gaben wir den Tieren am Tage der intraperitonealen Infektion und an den folgenden Tagen, und zwar INH in der Dosis von 10 mg/kg, Streptomycin in der Menge von 20 mg/kg.

Bestimmung des Sauerstoffverbrauchs. Den Sauerstoffverbrauch der Zellen ermittelten wir in dem WARBURGschen Apparat. In die einzelnen Gefäße wurden je 2 ml Zellsuspension, in die Seitengefäße 0,1 ml Alt-Tuberkulin oder PPD-Lösung eingemessen. (Im allgemeinen benutzten wir 5fach verdünntes Tuberkulin und 280 μg PPD.) Das Volumen wurde mit Ringer-Lösung auf ein Endvolumen von 2,5 ml ergänzt. Zur Kohlendioxydresorption benutzten wir 0,1 ml 20%iges NaOH. Nach Zugabe des Tuberkulins zu den Zellen wurden diese 10 Minuten inkubiert, dann die Röhren geschlossen und die Ablesungen in Intervallen von 10 Minuten vorgenommen. Aus der linearen Phase der 60 Minuten dauernden Versuche errechneten wir den Sauerstoffverbrauch von 100 000 Zellen während einer Stunde in μl auf Grund folgender Formel:

$$QO_{2S} = \frac{O_2\text{-Verbrauch} \times 100\,000}{\text{Zellzahl} \times 1\,\text{Stunde}}$$

Ergebnisse

Zuerst untersuchten wir die Wirkung von Tuberkulin auf die Asciteszellen der Meerschweinchen zu verschiedenen Zeitpunkten nach der Infektion. Die Versuche wurden 1, 3, 14 und 42 Tage nach der Infektion vorgenommen. Die Ergebnisse veranschaulicht Tabelle I. Hiernach verhalten sich die 3, 14 und 42 Tage nach der Infektion gewonnenen peritonealen Zellen in Anwesenheit von Tuberkulin ebenso wie die Zellen der nicht infizierten Kontrollmeerschweinchen, d. h. ihre Atmung wird von Tuberkulin nicht wesentlich beeinflußt. Erst 24 Stunden nach der Infektion zeigen die untersuchten Zellen Atmungssteigerung in Anwesenheit von Tuberkulin.

Um die Spezifität der festgestellten Reaktion zu klären, führten wir obige Versuche nicht nur mit Alt-Tuberkulin, sondern auch mit PPD durch, wobei derselbe Effekt zutage trat.

Tabelle I

Die Wirkung von Tuberkulin auf den Sauerstoffverbrauch der zu verschiedenen Zeitpunkten nach Tbc-Infektion gewonnenen Asciteszellen von Meerschweinchen

Versuch Nr.	Zellgewinnung nach der Infektion	I. QO_{28} ohne Tuberkulin	II. QO_{28} mit Tuberkulin	III. Steigerung in %
1	1 Tag später	0,084	0,107	27
2		0,096	0,132	38
3		0,090	0,117	30
4		0,082	0,102	24
5		0,091	0,124	36
1	3 Tage später	0,150	0,150	0
2		0,142	0,151	6
3		0,140	0,149	6
4		0,143	0,150	5
5		0,133	0,144	8
1	14 Tage später	0,142	0,153	8
2		0,123	0,153	16
3		0,132	0,135	10
4		0,127	0,145	14
5		0,115	0,123	7
1	42 Tage später	0,150	0,165	10
2		0,138	0,150	9
3		0,167	0,177	6
4		0,122	0,127	4
5		0,155	0,162	4
1	Gesunde Kontroll-gruppe	0,113	0,126	12
2		0,128	0,135	6
3		0,109	0,118	8
4		0,134	0,136	2
5		0,086	0,089	6

Signifikante Differenzen zwischen den Mitgliedern der I. Gruppe:
Zwischen der Kontroll- und 24stündigen Gruppe: $t = 6{,}0356$; $p > 0{,}001$
Zwischen der Kontroll- und 3tägigen Gruppe: $t = 4{,}187$; $0{,}01 > p > 0{,}001$
Zwischen der Kontroll- und 14tägigen Gruppe: $t = 1{,}264$; $0{,}3 > p > 0{,}2$
Zwischen der Kontroll- und 42tägigen Gruppe: $t = 2{,}9388$; $0{,}02 > p > 0{,}01$

Sämtliche in der Tabelle angeführten Versuche wurden mit den Zellen der vereinigten peritonealen Exsudate von drei Meerschweinchen vorgenommen. Die vereinigte Zellsuspension wurde in 4 Warburg-Gefäße verteilt. In zwei dieser Gefäße gaben wir 0,1 ml fünffach verdünntes AT. Das Endvolumen wurde mit Ringer-Lösung auf 2,5 ml ergänzt. Nach Zugießen von AT erfolgte die Inkubation der Zellen 10 Minuten mit offenem Manometer. Nach Verschließen des Manometers wurden die Versuche bei 37° C während 60 Minuten fortgesetzt und die Ergebnisse in Intervallen von 10 Minuten abgelesen. In der I. und II. Rubrik sind die ohne AT bzw. mit AT gewonnenen QO_{25}-Werte, in der III. Rubrik ist die Steigerung des Sauerstoffverbrauchs unter Wirkung von AT in % angegeben.

Im weiteren impften wir die Tiere auch mit avirulenten Mykobakterien ($H_{37}Ra$, BCG bzw. Saprophyten: *M. friburgensis*) und untersuchten die Atmung der Zellen 24 Stunden nach der Infektion in Anwesenheit von und ohne Tuberkulin: Atmungssteigerung kam auch bei diesen Versuchen nicht zustande.

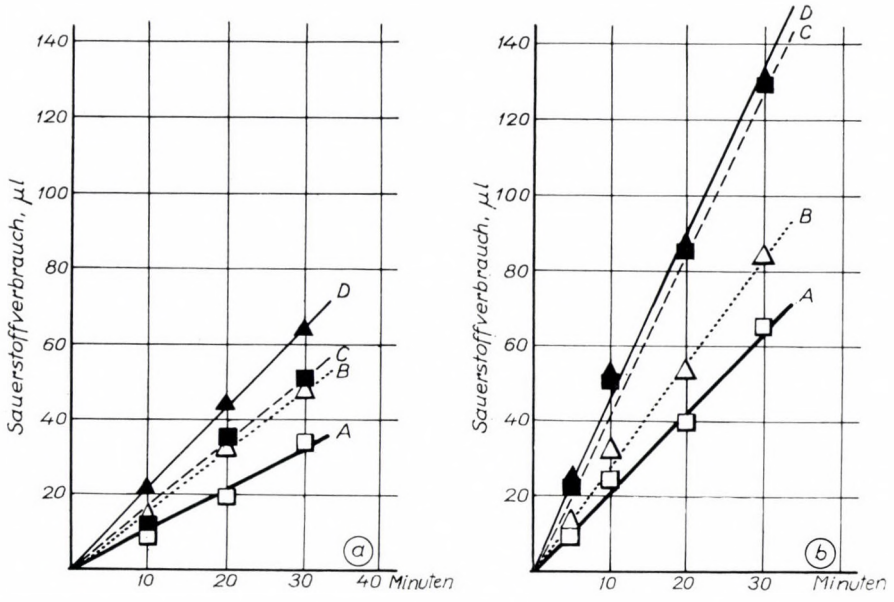

Abb. 1. Die Wirkung von Tuberkulin auf die Atmung der Asciteszellen von mit BCG immunisierten und superinfizierten Tieren (a) 1 Tag nach der Infektion, (b) 3 Tage nach der Infektion. Die Versuche wurden mit dem vereinigten peritonealen Exsudat von je 3 Meerschweinchen vorgenommen

(a) *Kurve A:* Kontrolle. Atmung der von den immunisierten und superinfizierten Tieren stammenden Zellen; Zellzahl $8{,}6\times10^6$
Kurve B: Mit der Kontrolle identisch $+0{,}1$ ml fünffach verdünntes Tuberkulin
Kurve C: Atmung der Zellen der mit 1 mg $H_{37}Rv$ infizierten Tiere 1 Tag nach der Infektion; Zellzahl $1{,}2\times10^7$
Kurve D: Dasselbe wie Kurve C + 0,1 ml fünffach verdünntes Tuberkulin

(b) Dasselbe wie unter Punkt a, aber 3 Tage nach der Infektion. Zellzahl bei den Kurven A und B $8{,}5\times10^6$, bei den Kurven C und D $1{,}5\times10^7$

Eine Atmungssteigerung vermochten wir auch dann nicht zu beobachten, wenn die Infektion zwar mit einem virulenten Stamm erfolgte, aber die Untersuchung der Zellatmung nicht 24 Stunden, sondern 20 Minuten nach der Infektion vorgenommen wurde.

Anschließend untersuchten wir, wie sich obige Erscheinung gestaltet, wenn die Tiere vorher mit BCG immunisiert bzw. vom Augenblick der Infektion an mit einem Bakteriostatikum behandelt werden.

Die mit BCG immunisierten Meerschweinchen infizierten wir intraperitoneal 6 Wochen nach der Immunisierung, dann wurden sie 1 bzw. 3 Tage

nach der Infektion getötet, wonach wir die Atmung der Asciteszellen nach der beschriebenen Methode mit und ohne Tuberkulin untersuchten. Abb. 1 enthält die Resultate.

Von BCG allein wurde die Atmung der Asciteszellen in Anwesenheit von Tuberkulin nicht beeinflußt. Geschah die Untersuchung 1 Tag nach der Infektion, so fanden wir, daß sich die Zellen der vakzinierten Tiere 1 Tag nach der Infektion ebenso verhielten wie die der nicht vakzinierten: ihre Atmung hatte in Anwesenheit von Tuberkulin zugenommen (Abb. 1*a*).

Ein auffallender Unterschied zeigte sich zwischen den vorigen beiden Tiergruppen, wenn die Untersuchungen 3 Tage nach der Infektion bzw. Reinfektion erfolgten: die Atmung der Zellen der vakzinierten Tiere war in Anwesenheit von Tuberkulin prägnanter gesteigert, während die der nicht vakzinierten keine wesentliche Abweichung zeigte (Abb. 1*b*).

Im Zusammenhang mit diesen sowie den 3 Tage nach der Infektion zutage tretenden Versuchsresultaten ergab sich ferner die Frage, ob der zellatmungsteigernde Tuberkulineffekt auch erhalten bleibt, wenn die immunisierten Meerschweinchen zuvor mit Tuberkulin desensibilisiert werden. Nach unseren Ergebnissen wird die Reaktion der Asciteszellen bei den immunisierten Meerschweinchen durch die Desensibilisierung mit Tuberkulin nicht verändert, wenn die Untersuchung 3 Tage nach der Infektion erfolgt.

Schließlich führten wir noch einen Versuch durch, bei dem die Tiere nach der Infektion bis zur Tötung mit INH bzw. Streptomycin behandelt wurden. Die Tötung der Tiere erfolgte 3 Tage nach der Infektion. Die Resultate sind in Abb. 2 zusammengefaßt.

Nach den Versuchsergebnissen war die Atmung der Zellen bei den mit INH behandelten Tieren gegenüber der bei den unbehandelten, aber infizierten Kontrolltieren 3 Tage nach der Infektion ebenso wie bei den mit BCG behandelten Tieren in Anwesenheit von Tuberkulin gesteigert. Demgegenüber wurde das Verhalten der Zellen in Anwesenheit von Tuberkulin durch die Streptomycinbehandlung 3 Tage nach der Infektion nicht beeinflußt.

Besprechung

Für die infektiösen Erkrankungen ist es im allgemeinen charakteristisch, daß eine gewisse Zeit zwischen der Infektion und dem Erscheinen der Krankheitssymptome bzw. histopathologischen Veränderungen verstreicht. Während dieser Inkubationszeit gehen komplizierte und wenig bekannte mikrobiologische bzw. biochemische Geschehnisse vor sich. Im Rahmen vorliegender Arbeit suchten wir einen Einblick in den Mechanismus dieser Geschehnisse im Falle tuberkulöser Infektion zu gewinnen. Wir untersuchten die Atmung der Asciteszellen in Anwesenheit von Tuberkulin und fanden, daß die Atmung der Zellen 24 Stunden nach der Infektion unter Wirkung von Tuberkulin wesent-

lich gesteigert ist, während die Atmung der von gesunden Kontrolltieren stammenden Zellen von Tuberkulin nicht beeinflußt wurde. Ebenso war die Atmung in Anwesenheit von Tuberkulin unverändert, wenn die Untersuchungen nicht 24 Stunden, sondern 3, 14 bzw. 42 Tage nach der Infektion vorgenommen wurden. Diese Befunde bestärken die Versuchsergebnisse von MARKS und JAMES [8] sowie SANFORD und Mitarbeitern [13], die in späteren Perioden

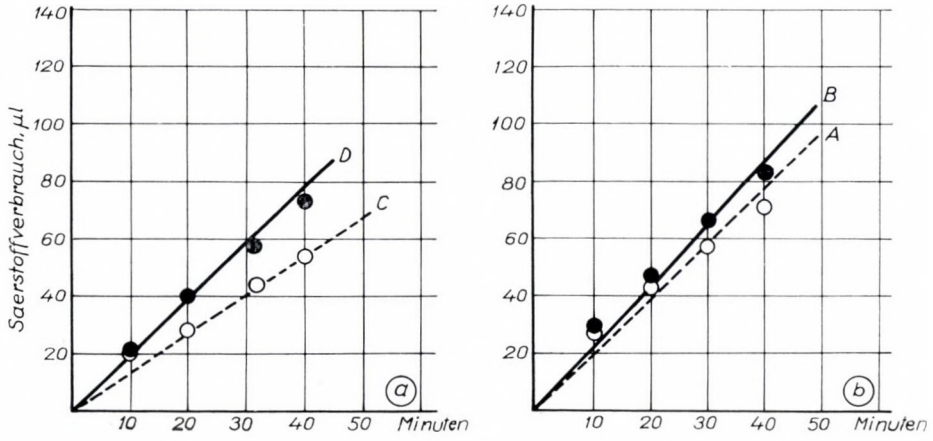

Abb. 2. Die Wirkung von Tuberkulin auf die Atmung der Asciteszellen der mit $H_{37}Rv$ infizierten und mit INH bzw. Streptomycin behandelten Tiere. Die Untersuchung geschah 3 Tage nach der Infektion

Die Versuche erfolgten mit den vereinigten Zellen von je 3 Tieren, die mit 10 mg/kg INH bzw. 20 mg/kg Streptomycin behandelt worden waren.

Kurve A: Sauerstoffverbrauch der von den infizierten und mit Streptomycin behandelten Tieren stammenden Zellen

Kurve B: Dasselbe wie A, jedoch in Anwesenheit von Tuberkulin. Zellzahl $6{,}6 \times 10^6$

Kurve C: Sauerstoffverbrauch der von den infizierten und mit INH behandelten Tieren stammenden Zellen

Kurve D: Dasselbe wie C, jedoch in Anwesenheit von Tuberkulin. Zellzahl $5{,}6 \times 10^6$

nach der Infektion unter Tuberkulinwirkung gleichfalls keine Atmungssteigerung beobachteten.

Nach unseren experimentellen Resultaten reagieren die peritonealen Exsudatzellen der infizierten Tiere in der Periode nach der Infektion verschiedenartig auf die Tuberkulinwirkung: 24 Stunden nach der Infektion ist ihre Atmung erhöht, während sich der atmungsteigernde Tuberkulineffekt nach 3 Tagen nicht manifestiert.

Im Zusammenhang mit diesen Versuchsergebnissen tauchen zwei Fragen auf: Kommt dem abweichenden Verhalten der Zellen irgendeine Bedeutung bei der Entwicklung der postinfektiösen Erkrankung zu? Worauf mag dieses abweichende Verhalten beruhen?

In bezug auf die erste Frage gelangten wir auf Grund unserer Resultate zu der Hypothese, daß das unmittelbar nach der Infektion nachweisbare

abweichende Verhalten der Zellen irgendeine Rolle bei der Entwicklung der späteren Erkrankung spielen müsse. Für diese Hypothese zeugen die Ergebnisse der Untersuchungen, die teils mit den mit BCG immunisierten und reinfizierten, teils mit den nach der Infektion mit INH behandelten Tieren durchgeführt wurden.

Nach diesen Versuchen wird die Atmung der Asciteszellen in Anwesenheit von Tuberkulin durch die Vakzination allein nicht beeinflußt (Abb. 1). Wurden die Tiere dagegen 6 Wochen nach der Vakzination mit virulenten Mykobakterien intraperitoneal superinfiziert, und untersuchten wir 24 Stunden später die Atmung der peritonealen Zellen in Anwesenheit von Tuberkulin, so sahen wir, daß sich die Zellen der vakzinierten Tiere 24 Stunden nach der Reinfektion ebenso verhielten wie die der nur mit virulenten Bakterien infizierten Tiere, d. h. ihre Atmung hatte in Anwesenheit von Tuberkulin zugenommen (Abb. 1*a*).

Ein auffallender Unterschied manifestierte sich indessen zwischen den Zellen der beiden Tiergruppen, wenn die Untersuchungen 3 Tage nach der Infektion bzw. Reinfektion vorgenommen wurden: die Atmung der von vakzinierten Tieren stammenden Zellen war in Anwesenheit von Tuberkulin prägnanter gesteigert, während die der nur infizierten Tiere keine Abweichung zeigte (Abb. 1*b*). Ein ähnliches Resultat sahen wir, wenn wir die Tiere unmittelbar nach der Infektion mit INH behandelten und die Zellatmung 3 Tage nach der Infektion untersuchten. Im Vergleich zu den unbehandelten, aber infizierten Tieren war die Atmung der von den mit INH behandelten Tieren stammenden Zellen in Anwesenheit von Tuberkulin gesteigert (Abb. 2).

Demnach weisen die Zellen, wenn der Organismus der Infektion gegenüber erhöhten Widerstand leistet (z. B. mit BCG immunisiert oder mit INH behandelt wurde), auch 1 bzw. 3 Tage nach der Infektion in Anwesenheit von Tuberkulin gesteigerte Atmung auf. Wird der Widerstand des Organismus nicht gesteigert, so tritt zwar 24 Stunden nach der Infektion unter Tuberkulinwirkung Atmungssteigerung auf, die jedoch 3 Tage nach der Infektion bzw. zu späteren Zeitpunkten infolge Progression der Infektion nicht mehr wahrgenommen werden kann.

Im Zusammenhang mit dem anderen Versuch stellte sich die Frage: Worauf kann das abweichende Verhalten der Zellen beruhen?

Aus den Versuchen von SEVER und YOUMANS [15] sowie anderer Autoren wissen wir, daß die Bazillenmenge in den ersten Tagen nach der Infektion unverändert bleibt. Nach den Ergebnissen ihrer Mäuselungenuntersuchungen erleidet die Bazillenmenge bis zum 4. Tage keine wesentliche Veränderung. Weiterhin stellten sie fest, daß die Vermehrungszeit der Mykobakterien in der Mäuselunge 25 Stunden, im Falle niedriger Dosen 29 Stunden beträgt. Laut ROBSON und Mitarbeitern [11] macht diese Zeitspanne 20,4 bzw. 21,9 Stunden aus. Aus diesen Angaben geht somit hervor, daß die Vermehrung der Myko-

bakterien erst nach einer gewissen Latenzzeit nach der Infektion einsetzt.

Vergleichen wir unsere Resultate mit den Literaturangaben, so scheint es möglich, daß die nach der Infektion wahrgenommene zellatmungsteigernde Tuberkulinwirkung mit der Latenzphase der Bakterien zusammenhängt. Hierfür sprechen auch die bei den mit BCG immunisierten bzw. mit INH behandelten Tieren erzielten Untersuchungsergebnisse. Nach den bereits erwähnten Versuchen von SEVER und YOUMANS [15] blieb nämlich die Bakterienzahl bei den immunisierten Mäusen nach der Infektion konstant. Andere Autoren [5, 9, 11, 12] vertreten ebenfalls die Meinung, daß sich die Schutzwirkung der Vakzination in einer Verzögerung der Vermehrung manifestiert. Ebenso wirkt auch INH: nach der Verabreichung sinkt jäh die Bakterienzahl, was schon beim Beginn der Bakterienvermehrung zur Geltung kommt [1, 7, 18].

Darauf läßt es sich vielleicht zurückführen, daß im Rahmen unserer Versuche der zellatmungsteigernde Tuberkulineffekt bei den vakzinierten bzw. mit INH behandelten Tieren auch 3 Tage nach der Infektion in Erscheinung trat. Nach der Vakzination bzw. INH-Behandlung kommt nämlich die Vermehrung der superinfizierten Bakterien nicht in Gang, wohl aber bei den nur infizierten Tieren, bei denen die Vermehrung der Bazillen postinfektiös nach einer kurzen Latenzperiode bereits einsetzt. Dies verursacht vielleicht das Ausbleiben der Tuberkulinwirkung auf die Atmung der Asciteszellen dieser Tiere.

Aus der Tatsache, daß unsere Versuchsergebnisse auch in denjenigen Fällen unverändert blieben, in denen die vakzinierten Tiere zuvor desensibilisiert wurden, darf womöglich geschlossen werden, daß die Allergie bei dieser Erscheinung keine Rolle spielt.

Schließlich ergab sich das Problem, ob der atmungsteigernde Tuberkulineffekt von der Phagozytose beeinflußt wird. Man könnte sich vorstellen, daß nach der Infektion die Vermehrung der phagozytierten Bakterien in den Zellen einsetzt, die Lebensfähigkeit der Zellen nachläßt und sich ihr Verhalten dem Tuberkulin gegenüber ändert, d. h. keine Atmungssteigerung zustande kommt. Für diese Möglichkeit sprechen die Resultate unserer Streptomycinversuche, bei denen wir feststellten, daß die nach der Infektion unter Tuberkulinwirkung eintretende Atmungssteigerung bei den Zellen der mit Streptomycin behandelten Meerschweinchen — im Gegensatz zu den Zellen der mit INH behandelten Tiere — nicht in Erscheinung trat. Sie verhielten sich demnach im wesentlichen so wie die Zellen der infizierten Kontrolltiere. Von Streptomycin wird angenommen, daß es auf die intrazellulär befindlichen phagozytierten Krankheitserreger keine Wirkung ausübt [6]. Demgegenüber diffundiert INH leicht in die Zellen, so daß es imstande ist, auch die Vermehrung der intrazellulär anwesenden Bakterien zu hemmen.

Daraus darf man vielleicht schließen, daß in erster Linie die intrazellulär anwesenden Mykobakterien für das Ausbleiben dieser Erscheinung

verantwortlich zu machen sind. Bei alledem handelt es sich naturgemäß nur um Hypothesen, die erst noch in weiteren Versuchen bewiesen werden müssen.

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ANTIGENIC STRUCTURE OF A NEW ENTEROPATHOGENIC E. COLI STRAIN

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Summary. It has been shown that the enteropathogenic organism isolated in 1959 and described as *E. coli* "Öskü" belongs to *E. coli* 0 group 143 and possesses an undetermined B antigen. Strain "Öskü" contains the complete antigen of *Sh. boydii* 8.

RÉDEY and CSIZMAZIA in 1959 showed that the guinea pig eye reaction was suitable for detecting and recognizing not only shigellae, but also other enteric organisms responsible for dysenteriform disease in man [1].

Applying this method they isolated, among other cultures, an organism which, on the basis of a positive keratoconjunctivitis reaction, was regarded as a pathogenic strain. The culture was obtained from the faeces of an 8 year old girl suffering from dysenteric symptoms. The organism was isolated on some additional occasions from the faeces of patients and symptomless carriers and from the water consumed by these persons.

Considering its biochemical reactions, the organism was classified into the Escherichia group and was named after the village where it had been first encountered as *E. coli* "Öskü" [1, 2].

Oral administration of the strain to volunteers [2] and a subsequent laboratory infection confirmed its pathogenicity.

The antigenic structure of the strain was not determined in the mentioned investigations. It was, however, shown that other *E. coli* strains associated with enteric disease (O groups 18, 26, 44, 55, 86, 111, 112*ac*, 119, 124, 125, 126, 127, 128 and 135) gave no reaction in an immune serum prepared with the incriminated culture. It was also shown that the strain was not related serologically to *Sh. dysenteriae* 1—7, *Sh. boydii* 1—7 and *Sh. sonnei* phase I and II [2].

In the present paper the detailed antigenic analysis of the organism is described.

Materials and methods

Cultures. E. coli "Öskü" 2064 (Rédey and Csizmazia 1959), *E. coli* type strains 0143, 015 and 0131 (State Serum Institute, Copenhagen) and type strain *Sh. boydii* 8 were used.

Immune sera. Serum "Öskü" was prepared by the method of EWING and EDWARDS [3]. For OK serum production an alcoholized suspension was used. *E. coli* grouping sera

01—0144 were prepared in the Institute of Microbiology, Pécs. Agglutination was tested first in 38 pooled sera containing each individual constituent at 1 : 100 dilution. The members of the polyvalent sera were chosen by considering the serum titres and antigenic relationships. K antigens were examined in Bl—B17 sera.

Biochemical reactions. Biochemical properties of strain "Öskü" were examined according to KAUFFMANN's method [4]. Amino acid decarboxylase reactions were determined as described by MØLLER [5]. Phenylalanine deaminase activity was shown by HENRIKSEN's method [6]. Motility was examined in U tubes containing semisolid agar (0.2 per cent).

Analysis of antigens. O antigen determination was carried out in pooled and subsequently in monovalent sera. The antigen was prepared by boiling the suspension of strain "Öskü" for 2 1/2 hours.

For K antigen determination the living strain was examined by slide agglutination in a set of OK sera representing 17 different B agglutinins.

Agglutinin absorption in O or OK sera was performed with bacteria grown in 24 hour liquid cultures. Prior to absorption the suspensions were boiled for 2 1/2 hours, then to the centrifuged cultures 1 : 50 diluted O sera or 1 : 20 diluted OK sera were added.

Results

Biochemical reactions. Repeated biochemical testing of strain "Öskü" yielded the following results. Nitrate reduction, indole production and the methyl red test were positive. Urea was not hydrolysed and ammonium citrate was not utilized. The KCN and Voges-Proskauer reactions were negative. Decarboxylase was produced for glutamic acid and ornithine, but not for lysine and arginine. Phenylalanine deaminase was not formed.

Acid and gas were produced in lactose, glucose, mannitol, galactose, laevulose, xylose, arabinose, rhamnose, raffinose, sorbitol, sorbose and trehalose. Maltose, dulcitol, salicin, adonitol, inositol and inulin were not fermented. Starch was not hydrolysed. The strain was non-motile. These reactions indicated that strain "Öskü" was a biochemically typical Escheric hia culture

Table I

Cross agglutination between strain "Öskü" and E. coli 0143

Sera	Titres with antigens			
	0143 100° C	0143 living	"Öskü" 100° C	"Öskü" living
0143 100° C	6400	800	6400	800
0143 living	1600	1600	1600	1600
"Öskü" 100° C	1600	100	1600	100
"Öskü" living	800	800	800	800

Analysis of antigens. Strain "Öskü" agglutinated in polyvalent serum No. 35. Of individual sera comprising this pool, the strain gave an end titre agglutination in serum 0143. In subsequent experiments cross agglutination reactions in O and OK sera prepared with strain "Öskü" and with type strain 0143 were performed by the use of boiled and living suspensions. The results are shown in Table I.

It is seen that the examined and the standard strains gave definite cross agglutination in all sera. The agglutination in O sera of living strains was inhibited. This inhibition was eliminated when heated cultures were tested. Cross absorption experiments with living and boiled cultures of the standard and examined strains are shown in Table II.

Table II

Cross absorption between strain "Öskü" and E. coli 0143

Sera	Cultures used for absorption	Titres with antigens			
		0143 100° C	0143 living	"Öskü" 100° C	"Öskü" living
0143 100° C	"Öskü" 100°	—	—	—	—
0143 100° C	"Öskü" living	—	—	—	—
"Öskü" 100° C	0143 100° C	—	—	—	—
"Öskü" 100° C	0143 living	—	—	—	—
0143 living	"Öskü" 100° C	—	—	—	—
0143 living	"Öskü" living	—	—	—	—
"Öskü" living	0143 100° C	—	—	—	—
"Öskü" living	0143 living	—	—	—	—

The results indicated the complete identity of O antigens. The finding that both boiled cultures were able to remove K agglutinins, pointed to the presence of a B-type K antigen. This antigen was not identified in the present studies. The examined strain did not agglutinate in our set of B sera (B1—B17).

As strain "Öskü" is non-motile, the question whether the organism is originally non-flagellated, can be answered only when further strains will be available.

A slight unilateral relationship is known to exist among O groups 15, 131 and 143. In addition, O group 143 is related to *Sh. boydii* 8 [7]. The result of cross-agglutination tests among *E. coli* 0143, 015, 0131, strain "Öskü" and *Sh. boydii* 8, and cross absorption tests among 0143, "Öskü" and *Sh. boydii* 8 is presented in Tables III and IV.

From Table III it is evident that in antigenic structure strains "Öskü" and 0143 are closely related to *Sh. boydii* 8. The relationship with strains 015 and 0131 is unilateral and unimportant. From the cross absorption tests presented in Table IV it is clear that in serological behaviour strains 0143 and "Öskü" are perfectly similar. Both *E. coli* strains possess a minor O factor, which is absent from *Sh. boydii* 8. In contrast, *Sh. boydii* 8 agglutinins are completely removed by both *E. coli* strains.

Table III

Cross agglutination among strain "Öskü", E. coli 0143, 015, 0131 and Sh. boydii 8

Sera	Titres with antigens				
	0143 100° C	"Öskü" 100° C	015 100° C	0131 100° C	*Sh. boydii* 100° C
0143	6400	6400	100	800	6400
"Öskü"	1600	1600	100	400	1600
015	—	—	1600	200	—
0131	—	—	50	3200	—
Sh. boydii 8	1280	1280	—	—	1280

Table IV

Cross absorption among strain "Öskü", E. coli 0143 and Sh. boydii 8

Sera	Cultures used for absorption	Titres with antigens		
		0143 100° C	"Öskü" 100° C	Sh. boydii 8 100° C
0143 100° C	*Sh. boydii* 8 100° C	100	100	—
"Öskü" 100° C	*Sh. boydii* 8 100° C	100	100	—
Sh. boydii 8	0143 100° C	—	—	—
Sh. boydii 8	"Öskü" 100° C	—	—	—

Discussion

E. coli strain 0143 was described by MATSUMOTO in 1958 [3] as a new species without any reference to its pathogenicity. The antigenic properties of the new O group were examined by ØRSKOV [7], who observed the mentioned minor serological relationship with O groups 15 and 131 and the strong cross reaction with *Sh. boydii* 8.

In the present study it has been shown that the enteropathogenic, biochemically typical *E. coli* strain "Öskü" corresponds serologically to the type strain of O group 143. As it follows from the lower agglutination titre of living suspensions, the strain contains a K antigen. Indicating the presence of B antigen, the absorptive capacity of heated suspensions remains unaltered. As the strain gives no reaction in the available OB sera, it probably contains a new B antigen. In antigenic structure, strain "Öskü" and the original 0143 culture are completely identical. Both cultures are only unilaterally related to groups O15 and 131. In contrast, there is an important relationship of the a—b variety between our strain and *Sh. boydii* 8. Antigen *a* is well-developed and represents the entire antigenic structure of *Sh. boydii* 8, while antigen *b* is the weakly developed own factor of the coli strains.

In recent years some biochemically and serologically typical *E. coli* strains have been isolated, which are capable of causing dysenteriform disease in man and keratoconjunctivitis in the guinea pig. Thus, as to pathogenic properties, the cultures behave similarly to shigellae. Organisms corresponding to the above criteria were found in *E. coli* O groups 25, 28, 32, 42 and 124. To this series now group 143 can be added. Strain 0135 isolated by RAUSS and VERTÉNYI probably also belongs to this group of pathogenic coli strains. With the latter organism, in lack of freshly isolated cultures, the guinea pig eye reaction was not examined. Such coli cultures contain the antigens of known or provisional Shigella serotypes [8—11]. Thus strain "Öskü" (0143) contains the complete antigen of *Sh. boydii* 8.

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BREAKDOWN OF AMINO ACIDS BY ENTEROBACTERIACEAE

II. STEREOSPECIFICITY OF ALKALIZATION REACTION

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Summary. The ability of a number of Enterobacteriaceae strains to split D, L and DL forms of alpha-alanine, serine, valine, tryptophan, histidine and glutamic acid, has been investigated. The various bacterium-amino acid combinations yielded highly variable results. Within one group (subgroup, type) in some cases all, or the majority, of strains, in others a smaller part of the cultures reacted stereospecifically. The complete absence of stereospecificity, when members of the examined group (subgroup, type) attacked uniformly all three isomers, was also observed. Within the same subgroup or type variable reactions were frequent. Thus from the data based on the examination of a small number of strains, no general conclusion can be drawn. The alkalization reaction in solutions of isomers of the same amino acid may be useful for diagnostic purposes.

In a previous paper [11] a method has been described for the routine examination of amino acid decomposition by enteric bacteria. By use of the same method, in the present study it has been investigated whether enteric bacteria are able to attack different stereo-isomers of the same amino acid uniformly, or the breakdown of these substances is stereospecific.

Materials and methods

Cultivation methods. Strains and methods for maintaining the cultures were as described in reference 10. In addition, Serratia strains kindly provided by *Dr.* E. ALDOVÁ, Institute of Epidemiology and Microbiology, Prague, were examined.

Preparation of media, technique of alkalization test and evaluation of results were described previously [11]. As amino acids L,D,DL-alpha-alanine, L,D,DL-serine, L,D,DL-valine, L,D,DL-tryptophan, L,D,DL-histidine and L and D-glutamic acid were used. Most of these substances were hydrochlorides manufactured by KETI or Reanal, Budapest.

In preliminary experiments the influence on alkalization of the size of inoculum was examined. From one day agar slant cultures of 5 *S. paratyphi B* and 5 *Sh. flexneri* 2a strains various numbers of bacteria (needle point, somewhat larger than needle point, one third and whole loopful — 3 mm loop — sized amounts) were transferred in three repeated experiments into 5 ml aliquots of a medium containing 0.2 per cent serine. In tubes inoculated with cultures picked up on the point of a needle no turbidity or alkalization developed during 6 days incubation. By increasing the inoculum, the number of alkalizing cultures also increased and the time needed for a shift of the indicator became shorter and there was a parallel increase in the reproducibility of the results. The best results were obtained when whole loopful cultures were suspended in the amino acid media. The results were similar when in subsequent experiments 10 Arizona, 10 Cloaca, 10 Klebsiella then 8 Arizona strains were tested in 0.1 per cent glycine, 0.1 per cent L-alpha-alanine, 0.2 per cent DL-ornithine or 0.1 per cent L-cysteine. Therefore in subsequent testing inoculations were performed by means of a loop (3 mm in diameter) fully packed with the culture.

Results

Breakdown of alpha-alanine stereo-isomers. Alkalization by 354 strains was examined in pH 4.7—4.9 alpha-alanine solutions. Although parallel experiments in 0.1 and 0.2 per cent solutions often yielded uniform or at least similar results, the DL amino acid was used at 0.2 per cent concentration. In the latter solution alkalization was more rapid and definite.

The reactions in alpha-alanine isomers are presented in Table I. Organisms that were unable to attack either L or D isomers (15 *P. vulgaris*, 14 *P. mirabilis*, 15 Morganella, 13 Rettgerella, 15 Providencia, 14 *Sh. dysenteriae*, 10 *Sh. flexneri* 3, 13 *Sh. boydii)* were omitted. From Table I it is clear that with alpha-alanine stereospecificity was rarely observed. Klebsiella, Citrobacter, Cloaca, Serratia, *S. paratyphi B*, *S. typhi*, Arizona and *Sh. sonnei* strains unexceptionally attacked all isomers. Among other enteric bacteria only a small number of organisms *(Sh. flexneri)* occurred which deaminated the L form without acting on the D isomer.

Some bacteria *(S. typhi, Sh. sonnei)* seemed to decompose the racemic form more intensively than either the D or the L isomers. This finding may be explained by the higher concentration of DL solution (0.2 per cent). It is

Table I

Alkalization by enteric bacteria in alpha-alanine
5 ml 0.1% L, or D-alpha-alanine or 0.2% DL-alpha-alanine, pH 4.7—4.9, loopful inoculum

Group (subgroup, type)	No. of strains	Percentage of positive cultures* in alpha-alanine solutions after incubation periods of								
		1 day			3 days			6 days		
		L	D	DL	L	D	DL	L	D	DL
E. coli	45	27	9	24	98	69	95	98	87	100
E. coli 0124	20	0	0	0	70	5	95	80	25	95
Klebsiella	15	100	80	80	100	100	100	100	100	100
Citrobacter	15	7	7	27	100	100	100	100	100	100
Cloaca	15	73	47	73	100	93	100	100	100	100
Serratia	5	100	100	100	100	100	100	100	100	100
S. paratyphi B	18	0	5	11	100	80	100	100	100	100
S. typhi murium ...	46	15	0	11	100	50	100	100	91	100
S. typhi	14	0	0	0	85	85	100	100	100	100
Arizona	15	33	40	20	100	100	100	100	100	100
Sh. flexneri 2	28	4	0	0	43	0	32	79	0	86
Sh. flexneri 4, 5, 6	13	0	0	0	0	0	31	23	0	69
Sh. sonnei	10	10	0	0	70	40	100	100	100	100

* Weak positive results (±) have been included in the positive reactions

known that when the substrate concentration is increased, the amount of decomposition products, that is, ammonia, also increases to a certain limit

With the exception of the Proteus-Providencia group, similar results were obtained when alkalization was examined in phenol-red-containing solutions of alpha-amino acid isomers adjusted to pH 6.5. Proteus-Providencia bacteria were able to attack all three forms at higher pH values. Under these circumstances the stereospecificity of the reaction for other bacteria increased: some *S. paratyphi B* strains decomposed only L (and DL) substances but not the D form. In more alkaline media (pH 7.3 with m-cresol purple indicator) stereospecificity further increased.

Breakdown of serine stereo-isomers. The results obtained with 190 strains (Table II) indicate that in the medium with the initial pH 4.8 this reaction was only exceptionally stereospecific. Of *E. coli* strains 2, of Morganella strains 22 decomposed only L-serine, while 5 *S. typhi* strains only D-serine.

Breakdown of valine stereo-isomers. Decomposition of valine stereo-isomers was examined with Enterobacteriaceae strains listed in Table II. In addition, 10 Serratia strains were tested. In media adjusted to pH 4.9 all bacteria but Serratia strains failed to alkalize. Of 10 Serratia strains 2 attacked the L isomer, 5 the DL mixture. In D-valine all cultures were inactive.

Breakdown of tryptophan stereo-isomers. In pH 4.9 tryptophan solution, 135 Enterobacteriaceae strains were tested. Active strains were encountered only in the *E. coli* and Serratia groups. Of 29 *E. coli* cultures 23 attacked L and DL tryptophan; neither of the cultures acted upon the D isomer. Thus these organisms gave uniformly a stereospecific reaction. All the 7 examined Serratia strains attacked L and DL-tryptophan; in D-tryptophan only 2 cultures caused alkalization. Rettgerella and Providencia cultures behaved differently. These organisms eventually caused in pH 4.9 solutions, too, a cherry-red coloration characteristic of the iron salt reaction indicating tryptophan deaminase activity [12]. This phenomenon will be detailed in a subsequent paper. The reaction was strictly specific; it never occurred in D-isomer solutions.

Breakdown of histidine stereo-isomers. Due to a shortage in D-histidine, only 93 of the listed Enterobacteriaceae strains were examined. A seemingly stereospecific alkalization was observed with the used 5 Providencia cultures, which attacked only L and DL substances. Other positive results (5 of 5 Klebsiella, 1 of 5 Citrobacter, 3 of 8 Cloaca and 4 of 4 Serratia strains) appeared in all histidine isomers.

Breakdown of L and D glutamic acid. As seen in Table III, the majority of the 176 examined cultures attacked both isomers *(E. coli*, Klebsiella, Citrobacter, Cloaca, Serratia, *S. paratyphi B*, *S. typhi murium*, Arizona, Providencia). About 50 per cent of Rettgerella strains decomposed only the L form, some *P. vulgaris* cultures only the D form.

Table II

Alkalization by enteric bacteria in serine
5 ml 0.1% L, or D-serine or 0.2% DL-serine, pH 4.8, loopful inoculum

Group (subgroup, type)	No. of strains	Percentage of positive cultures* in serine solutions after incubation periods of								
		1 day			3 days			6 days		
		L	D	DL	L	D	DL	L	D	DL
E. coli	10	0	10	0	100	80	100	100	80	100
Klebsiella	10	20	70	50	100	100	100	100	100	100
Citrobacter	10	0	10	10	100	100	100	100	100	100
Cloaca	10	10	10	30	100	100	100	100	100	100
Serratia	5	60	40	80	100	100	100	100	100	100
S. paratyphi B	10	0	0	0	100	100	100	100	100	100
S. typhi murium ...	10	0	0	0	100	100	100	100	100	100
S. typhi	25	4	0	0	52	64	76	56	72	84
Arizona	15	0	0	0	44	13	27	100	13	47
Sh. flexneri 2	10	0	0	0	0	0	0	0	0	0
P. vulgaris	10	0	0	0	0	0	0	0	0	0
P. mirabilis	10	0	0	0	0	0	0	0	0	0
Morganella	35	0	0	34	80	0	77	86	0	77
Rettgerella	10	0	0	0	0	0	0	0	0	0
Providencia	10	0	0	0	0	0	0	0	0	0

* Weak positive results (±) have been included in the positive reactions

Discussion

The characteristic substrate specificity of enzymes is valid also for optical isomers. The stereospecificity of amino acid-decomposing bacterial enzymes has been confirmed by numerous data. Bacterial proteins almost unexceptionally consist of L amino acids. D amino acids have been revealed only as rare exceptions, *e.g.* in antibacterial peptides produced by some Bacillus species. Of alanine stereoisomers the bacterial cytoplasm contains the L form. In synthetic DL-alanine bacteria can use only the L isomer and leave the D form almost completely unattacked. Similarly, lysine decarboxylase produces cadaverine and CO_2 only from L-lysine [6]. Amino acid oxidases are also substrate specific and oxidize to ketones either the L or the D isomer [5]. *P. vulgaris*, *Ps. aeruginosa* and *A. aerogenes* deaminases are able to produce ammonia only from the L-isomers of 11 different amino acids [13]. *P. vulgaris* aspartase responsible for the decomposition of aspartic acid into fumaric acid and ammonia, is strictly stereospecific [8]. The same holds true for the glutamic acid dehydrogenase of *Neurospora crassa* and for streptococcal tryp-

Table III

Alkalization by enteric bacteria in glutamic acid
5 ml L or D-glutamic acid, pH 4.7, loopful inoculum

Group (subgroup, type)	No. of strains	Percentage of positive cultures* in glutamic acid solutions after incubation periods of					
		1 day		3 days		6 days	
		L	D	L	D	L	D
E. coli	15	0	0	93	80	100	100
Klebsiella	10	50	30	100	100	100	100
Citrobacter	15	0	0	67	73	100	100
Cloaca	5	100	40	100	100	100	100
Serratia	5	40	0	100	100	100	100
S. paratyphi B	10	0	0	40	10	100	100
S. typhi murium	10	0	0	75	19	100	100
S. typhi	10	0	0	0	0	0	0
Arizona	10	0	0	80	80	100	100
Sh. flexneri 2	18	0	0	0	0	0	0
P. vulgaris	13	0	0	31	31	54	31
P. mirabilis	10	0	0	0	0	0	0
Morganella	13	0	0	0	0	0	0
Rettgerella	13	8	8	23	46	46	77
Providencia	13	0	0	62	77	85	85

* Weak positive results (±) have been included in the positive reactions

tophan decarboxylase [4]. *E. coli* deaminates the L isomers of alanine and glutamic acid [6].

Among the numerous observations suggesting the stereospecificity of enzymes responsible for microbial amino acid metabolism, there are data as to the occurrence of an occasional limited specificity. Although in synthetic DL-alanine the L isomer is usually utilized, recent studies have revealed that growing cells are able to take up small amounts of D-alanine [6]. The Proteus group attacks only the L form of most amino acids, but it is able to utilize both isomers of alpha-alanine and serine [2]. The deaminase of *E. coli* acts upon both optical isomers of serine [3]. The aspartic acid deaminase of *Aspergillus niger* is not specific for either of the isomers of the substrate [7]. As to the metabolism of *Ps. aeruginosa*, the amino acids can be divided into 3 groups. Against amino acids of group 1 (leucine, isoleucine and histidine) there is a strict stereospecificity, as the corresponding enzyme attacks only the natural isomer. In group 2 (valine and phenylalanine) presumably the D isomer is oxidized without deamination, or the non-natural isomers may

promote residual respiration. Finally, *Ps. aeruginosa* attacks both optical isomers of alanine, serine, tyrosine and proline [14].

The interpretation of these partly contradictory data is difficult, as most of them have been yielded by classical biochemical investigations. The procedures employed in enzyme research (use of lysates or repeatedly washed cultures, incubation of the enzyme with the substrate, quantitative determination of products, or serial measurements in the Warburg apparatus) are delicate and laborious. This explains the fact that the available data on the stereospecificity of enzymes are based mainly on experiments with one or two strains. The alkalization test cannot be compared in exactness with the above delicate methods; nevertheless, it is advantageous in that it allows the examination of a series of different strains. The present investigation revealed that even strains belonging to the same serotype may react differently with amino acid stereo-isomers. Thus the stereospecificity of the enzymes produced by a given group of bacteria can only be estimated when a large number of strains is investigated.

Conclusions as to the diagnostic validity of the alkalization test are not justifiable. From our experiments it is clear that the presence or absence of alkalization in various stereo-isomers may be characteristic of certain bacteria. Thus of Shigellae, D-alpha-alanine was attacked only by *Sh. sonnei;* Morganella strains may decompose L-serine but not the D form; *E. coli* deaminates only the L isomer of tryptophan.

In examining the breakdown of amino acids it is usual to use the L isomer at 0.1, and the DL mixture at 0.2 per cent concentration. The 0.2 per cent solution of the racemic compound is equivalent to the 0.1 per cent L solution, when the microorganism does not act upon the D form. When both optical isomers are decomposed, the 0.2 per cent solution of DL amino acid contains a twofold amount of substrate. As shown by experiments with alpha-alanine, the concentration of the substrate influences the result. The 0.1 per cent L and 0.2 per cent DL solutions are generally regarded as equivalent. However, when the tested organism attacks only the D amino acid, it reacts differently in 0.1 per cent L and in 0.2 per cent DL solutions, as in the former it gives a negative, in the latter a positive, result.

There is some difference between the data reported in the present and our previous paper [11]. Apart from insignificant aberrations, alpha-alanine decomposition by *S. typhi* must be mentioned. This activity had not been observed previously, while it was definite in the present experiments. In our opinion the difference should only partly be attributed to the slightly higher pH of the medium. The main reason for this behaviour might be the difference in the number of inoculated bacteria. As pointed out in the part on "Materials and methods" of the present paper, seeding with bacteria picked up on the point of a needle often yielded negative results.

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SIGNIFICANCE OF HAEMAGGLUTINATION-INHIBITING ANTIBODIES IN THE EVALUATION OF VACCINIAL REACTIONS

By

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Summary. The relationship between prevaccination haemagglutination-inhibiting (HAI) titre and reaction to smallpox revaccination has been studied. The chance of take for subjects with titres under 1 : 4 was higher than for those with titres 1 : 4 or higher. Attempts have failed to establish a HAI antibody level sufficient to prevent the take with a high probability. Failures to take may not be explainable by the HAI antibodies of the serum.

Successful vaccination is regularly accompanied by a rise in the serum HAI antibody level. Demonstration of the HAI antibody response may be of help in differentiating takes from failures.

The general opinion on the duration of immunity following smallpox vaccination has changed since it had become obvious that life-long immunity is not provided by this vaccination either. Susceptibility, which seems to be in direct relation to the time elapsed since the last vaccination, returns within 10 years [1]. Examination of the susceptibility of revaccinated subjects is based on the vaccinial reaction. Earlier it was assumed that an unsuccessful vaccination is indicative of prevailing immunity and *vice versa*, successful vaccination means susceptibility. This view manifests itself in the issue of international vaccination certificates.

Meanwhile, however, data have accumulated evidencing that this simple way of evaluation is incorrect; *e.g.* numerous subjects of those having introduced smallpox into Europe had been considered immune on the basis of an unsuccessful vaccination performed shortly before.

A number of experiments have shown that the vaccinial reaction is greatly influenced by the vaccination technique [2] and the quality of the vaccine [3].

These observations have changed the explanation of the significance of vaccinial reaction. Now the evaluation of the reaction is based on the following principles.

(i) Successful vaccination (take) means a primary or accelerated (vaccinoid) reaction with pustule-formation. This is accompanied by the development of complete immunity to smallpox.

(ii) Unsuccessful vaccination may be classified into two groups.

(a) "No take", *i.e.* lack of tissular reaction, may indicate immunity in freshly-immunized subjects; otherwise it results from technical failure.

(b) Immediate (earlier: immune) reaction. The tissular reaction consists of erythema and induration; the areola is largest between 8 and 72 hours. There is no pustule even a vesicle rarely appears. This reaction may be shown by really immune subjects, but susceptible persons may elicit the same reaction as a result of technical failure or use of a detoriated vaccine. Susceptible subjects may develop a primary or an accelerated reaction if they are revaccinated on one or more occasions.

Several problems arise in connexion with unsuccessful vaccination. A "no take" or immediate reaction gives no information on the vaccinee's immune status, although knowledge of the immune status would be important if there is a risk of smallpox infection.

Thus, another question arises, *viz.* how many unsuccessful revaccination attempts provide reliable evidence of immunity against smallpox.

We wished to approximate the question by completing our vaccination studies with serological examinations. It was assumed that repeated failures to take are due to an immunity which is demonstrable serologically, and this assumption raises a further question, *viz.* whether there exists an antibody level which is sufficiently high to prevent the take and, consequently, makes further attempts of vaccination unreasonable.

Of the available serological reactions we chose the haemagglutination-inhibition (HAI) test. NAGLER was the first to report on the appearance of HAI antibodies after attacks of smallpox and vaccinations against smallpox. The specificity of the test has been proved by several authors [5—9].

Materials and methods

Serum samples. Blood was taken from 20—60 years old adults before revaccination (in the following "vaccination" means revaccination) and 3—4 weeks thereafter. The serum specimens were inactivated at 56° C for 30 minutes and stored in the frozen state.

Vaccine. The Hungarian commercial vaccine (bovine dermovaccine) was used. The vaccine contained 7×10^6 pock-forming units as titrated on the chorioallantoic membrane of the 12-day-old chick embryo.

Vaccination technique. The skin was scarified in the upper lateral third of the arm through a drop of vaccine placed on the skin before scarification. Initially a double-cross was formed; after several unsuccessful attempts four horizontal and four perpendicular scratches 6—8 mm long were made. In the case of a "no take" or "immediate" reaction vaccination was repeated at least on one occasion, in a few cases even five times.

Evaluation of vaccinial reactions. The reaction was read on the 7th day after vaccination. Primary and accelerated reactions were considered successful, "no takes" and immediate reactions unsuccessful. The reaction was regarded as doubtful when we were unable to decide whether the crust, being 2—3 mm in diameter, had derived from a pustule or an allergic vesicle. (In the latter case the course of the reaction would have provided some further information; however, most of the vaccinated persons could not give adequate information.)

HAI test. This was carried out as recommended by SZATHMÁRY and HOLIK [10], essentially as follows. A twofold serum dilution series was prepared in TAKÁTSY's [11] Microtitrator apparatus and 2 haemagglutinating units of the egg-adapted vaccinia virus were added to every dilution. The serum-virus mixtures (0.05 ml) were incubated at 37° C for one hour, and then 0.025 ml of 1-per-cent suspension of chicken erythrocytes agglutinable with vaccinia virus was added. The results were read after incubation at room temperature for 30 minutes. The HAI titre was expressed as the highest dilution completely inhibiting haemagglutination. Titres of 1 to 4 or higher were accepted as positive.

Experimental

(i) *Relationship between prevaccination HAI titre and vaccinial reaction.* Twohundred seventytwo subjects were divided into two groups on the basis of their prevaccination titres (Fig. 1, columns 1 and 2). The percentage of takes was higher in the seronegative group than in the subjects with prevaccination titres 1 : 4 to 1 : 32 (51.6 per cent and 39.3 per cent, respectively). The percentage of doubtful reactions was approximately the same in the two groups (8.4 and 9.4 per cent).

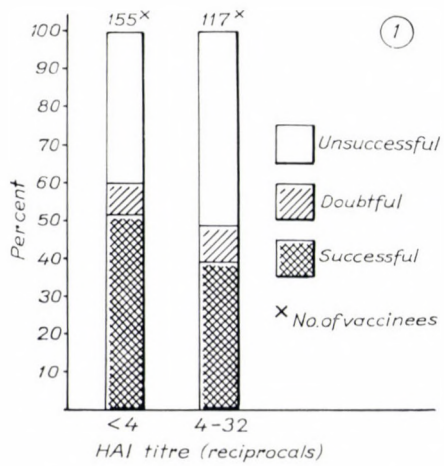

Fig. 1. Relationship between prevaccination HAI titre and result of first vaccination attempt

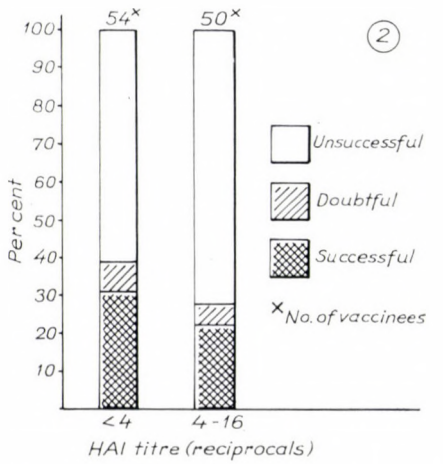

Fig. 2. Relationship between prevaccination HAI titre and result of second vaccination attempt (Data of persons showing no take at first attempt)

In Fig. 2 the relationship between prevaccination HAI titre and vaccination result is illustrated on the basis of the reactions of 104 subjects whose vaccination had been unsuccessful at the first attempt. Here again, the chance of take was somewhat higher in the seronegative subjects than in those with prevaccination titres as high as 1 : 4 to 1 : 16 (31.4 and 22.0 per cent, respectively).

Table I shows the prevaccination titres of 127 subjects with a take on the first revaccination attempt.

In Table II the prevaccination titres of those 32 persons are presented who could not be revaccinated with success even by three or more attempts. The distribution of these titres is not markedly different from that of the titres of unselected sera, although formerly these subjects would have been regarded to be immune.

Table I

Prevaccination HAI titres in the sera of subsequently successfully vaccinated persons at time of first attempt

HAI titre (reciprocals)	<4		4		8		16		Total	
	No.	%	No.	%	No.	%	No.	%	No.	%
Distribution by HAI titre	81	(63.8)	22	(17.3)	20	(15.8)	4	(3.1)	127	100

Table II

Prevaccination HAI titres in the sera of subjects subsequently vaccinated three or more times without successs

HAI titre (reciprocals)	<4	4	8	16	Total
Distribution by HAI titre	16	5	10	1	32

(ii) *Relationship between vaccinial reaction and HAI antibody response.* Paired sera were examined from 168 vaccinees. The relationship between vaccinial reaction and HAI antibody response is illustrated in Figs. 3—5.

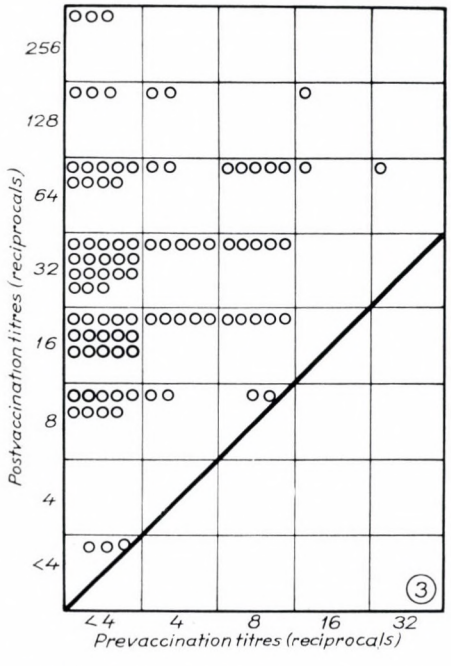

Fig. 3. HAI titres before and after successful revaccination

(a) Successful vaccinations (Fig. 3). Out of 95 takes 90 were accompanied by HAI antibody response. The postvaccination titres did not surpass 1 : 256.

(b) Unsuccessful vaccinations (Fig. 4). Out of 44 unsuccessful attempts only 8 were followed by a slight rise in the HAI titre. The increase was only in two cases four-fold or greater.

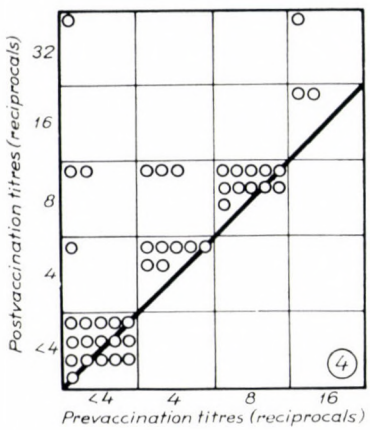

Fig. 4. HAI titres before and after unsuccessful revaccination

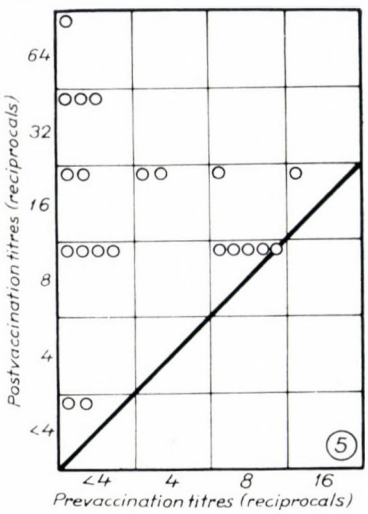

Fig. 5. HAI titres before and after revaccination with doubtful result

(c) Vaccinations with doubtful success (Fig. 5). Eight of the doubtful reactions were not followed by any rise of the HAI antibody level.

Discussion

We wish to discuss two questions on the basis of the present results.

(1) Is there any correlation between the antibody content and the success of revaccination in subjects vaccinated years ago?

(2) The significance of the HAI test, especially as regards the reasonability of revaccination of subjects at high risk of smallpox infection.

The subjects under study were adults who had been revaccinated more than 10 years earlier. Thus, they may have had lost their immunity or their protection had fallen to a rather low level. It is, however, well-known that in such a population there are subjects who do not take revaccination, *i.e.* appear to be protected, although their specific reaction to repeated revaccination will prove their susceptibility. In such cases the question arises as to how many times revaccination should be repeated. PARISH and CANNON [12] recommend two repetitions, *i.e.* they think that three successive failures to take indicate a solid protection.

In the course of the present studies, however, the 4th, 5th or even 6th attempts were followed by a take in numerous cases and the HAI titres of these subjects were not higher than those of persons who gave a primary reaction on the first occasion (Tables I and II). The data in Figs. 1 and 2 suggest that there may be some parallelism between HAI titre and protection as indicated by the vaccinial reaction, but the quantitative value of the HAI titre provides no adequate information on succeptibility or immunity. Although in seronegative subjects (HAI $< 1 : 4$) the percentage of successful vaccination was higher than in those possessing HAI antibodies, we were unable to find a threshold titre above which successful revaccination cannot be expected.

HOLIK and SZATHMÁRY [7] assume that subjects with HAI titres 1:64 or higher cannot be vaccinated with success. Unfortunately, in the present material such high prevaccination titres did not occur. On the other hand, we do not agree with SERENKO [13], who regards the HAI titres exceeding 1 : 10 to indicate protection.

The question as to whether the presence or absence of HAI antibodies provide information on the immune status has not yet been answered.

The importance of HAI antibodies is supported by their specificity. They regularly appear after smallpox attacks as well as successful vaccinations and, according to SERENKO [13], the titres gradually decline after revaccination. It is in accordance with this view that our vaccinations were more successful in the seronegative group (Fig. 2).

On the other hand, according to COLLIER [5], the HAI antibodies disappear as soon as one to three years after vaccination, when immunity is still expected to persist. In DOWNIE's [14] opinion the HAI antibodies of the serum disappear earlier than the neutralizing antibodies.

The findings of SZATHMÁRY and BARANYAI [15] also raise some doubt as regards the importance of HAI antibodies. These authors were not able to confirm SERENKO's [13] observation on the correlation between age and HAI titre.

The importance of HAI antibodies as indicators of immunity to smallpox is, therefore, questionable. The morbidity rate of persons with different HAI titres would be the only firm basis for adequately answering this question. Since such data are lacking, it seems reasonable to repeat revaccination of highly-risked persons until the specific (primary or vaccinoid) reaction has been achieved. In the course of the revaccination of 855 employees of the Central Hospital for Infectious Diseases we achieved takes in nearly 100 per cent by 5 or 6 attempts.

A significant rise of the HAI titre is an appropriate indicator of the multiplication of vaccinia virus in the organism (Fig. 3), as shown by literary

data and the present investigations. Unsuccessful vaccination is only rarely followed by HAI antibody response (Fig. 4).

It should be emphasized that in a number of cases the doubtful reaction was not followed by HAI antibody response (Fig. 5), *i.e.* immunization failed to take place.

Populations exposed to special risk of smallpox, *e.g.* the personnel of infectious hospitals, should be immunized as effectively as possible. When the vaccinial reaction is doubtful, the HAI antibody may provide additional information. For this purpose blood should be taken at the time of observing the doubtful reaction, and another sample 2 or 3 weeks later. Since according to literary data the HAI titre does not begin to rise within a week after revaccination, a significant rise in titre may be accepted as the indicator of successful vaccination.

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FERMENTATION BY SYNCHRONOUSLY DIVIDING YEAST CELLS IN NITROGEN-POOR MEDIUM

By

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Summary. The rate of fermentation exerted by synchronously dividing yeast cells has been found to increase intermittently. In the fermentation curve periodic declines coinciding with the budding of cells could be observed. The declines were eliminated by the addition of inorganic nitrogen salts. The effect varied in degree with the nitrogen content of the yeast preparation; at higher incubation temperatures it ensued more rapidly. No similar effect has been observed in the increase of the respiration rate. From the results it has been concluded that yeast cells multiplying in a nitrogen-poor medium unsuitable for supplying the increased nitrogen requirement, may be able to decompose members of their own carbohydrate-splitting enzyme system.

The amount of carbon dioxide produced in complete media by yeasts at given intervals, increases parallel with the increase of the living cell count until the sugar content of the medium has decreased to a certain concentration. Measurements in the Warburg apparatus have shown that in molasses-phosphate salt medium free from inorganic nitrogen, the continuous increase in the rate of fermentation ceases in certain periods, or occasionally even the absolute intensity of fermentation decreases [1]. The effect appears at definite periods and coincides with the budding of the cells. The purpose of the present experiments was to define conditions responsible for this finding.

Materials and methods

In the experiments baker's and seed yeast produced by Department 2, Budapest Yeast Factory, were used. The industrial term for Baker's yeast is "generation V" and that for the seed yeast used for seeding the mash in which baker's yeast is produced, "generation IV". No particular method was applied for synchronizing the cultures, as at the first division about 80 per cent, at the second division about 60 per cent of the cells were in the same phase. This degree of synchrony was found satisfactory for the experiments. OGUR *et al.* were the first to observe that, when measured by the percentage of budding cells, the synchrony of baker's yeast was high in the first division cycle [2]. We made similar observations in industrial seed yeast cultures [3]. Synchrony was estimated by determining the percentage frequency of budding cells. The cultures were not subjected to synchronization, as the generally applied methods [4—6] were not delicate enough to exclude errors resulting from the synchronizing procedure proper.

Intensity of fermentation and respiration was measured in the Warburg apparatus as follows. Yeast samples of 0.05—0.4 g were suspended in 100 ml phosphate buffer. The amount of yeast was chosen within the above limits so as to yield the highest amount of gas without exhausting the total sugar content of the medium. Depending on the reaction to be observed, the experiments lasted for 4 to 18 hours at 30° C. The phosphate buffer (pH 4.9) was prepared by mixing 990.1 ml solution A (9.078 g KH_2PO_4 in 1000 ml) with 9.9 ml solution

B (11.876 g $Na_2HPO_4 \cdot 2\ H_2O$ in 1000 ml). In the central well of the Warburg flask was placed 2.0 ml yeast suspension, in the side arm 0.25 ml 10 per cent sucrose-containing molasses medium, or in some experiments 0.25 ml 10 per cent sucrose solution. Varying with different samples, the pH values of the molasses solutions were between 5.3 and 5.5. The experiments were performed with molasses obtained from various sugar factories (Hatvan, Selyp, Szerencs and Sarkad). In order to supply oxygen for multiplication, the Warburg flasks were not flowed through with nitrogen. Due to the Pasteur effect, this would have resulted in a more intensive fermentation. In the measurements, oxygen uptake was not considered, as the RQ. was 1, and the difference in volume attributable to the different solubility of CO_2 and O_2 was less than 1 per cent of the total amount of CO_2 produced in fermentation. At the initial stage the difference was somewhat larger (not more than 3 to 4 per cent), this stage, however, was not important for the evaluation of the results. Thus in the graphs "μl CO_2" indicates practically the amount of CO_2 produced during fermentation. The manometers were read at 10 minute intervals. In the graphs the respective amounts of CO_2 and O_2 produced and consumed, were plotted against time.

Results

As measured by the described method, the intensity of respiration increased continuously and proportionally. In contrast, the increase in the intensity of fermentation was not continuous. Varying with the yeast preparation, after 3 to 4 hours a decline appeared in the fermentation curve. Between the 6th and 8th hours a subsequent, more definite decline was observed. At the first decline the increase in the rate of fermentation ceased, but the absolute value of fermentation intensity remained unaltered. At the second decline, absolute fermentation intensity also decreased definitely (Fig. 1, curve "Generation V").

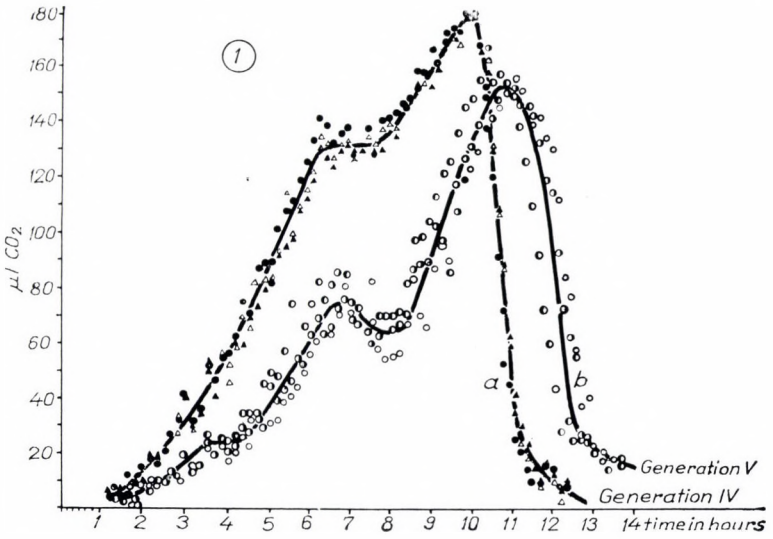

Fig. 1. Fermentation by synchronously budding yeast culture in molasses-phosphate medium. The points obtained in 3 parallel experiments are shown separately. Yeast concentration, 0.05 g/100 ml buffer

It was striking that unlike to baker's yeast, with the seed yeast culture the first decline failed to appear and the second was less marked (Fig. 1, curve "generation IV").

It was supposed that the difference between the two cultures was associated with their different nitrogen content. The nitrogen content of baker's yeast was 6.2 per cent, that of the seed yeast 6.7 per cent by dry weight. Therefore the effect of inorganic nitrogen compounds was examined. At first 0.4 per cent ammonium sulphate was added to the phosphate buffer. The obtained respiration and fermentation activities are shown in Fig. 2. It can be seen that the rate of fermentation and respiration increased continuously without the declines shown in Fig. 1. Then various amounts of ammonium sulphate were added to the phosphate buffer and the rate of fermentation was examined under the same conditions. The result is presented in Fig. 3. It is evident that the gradually increasing concentrations of nitrogen delayed the appearance and decreased the size of declines. The minima appeared more or less at the same intervals. The nitrogen supplement, of course, increased the absolute value of fermentation rate. The presence of 0.1 per cent or more ammonium sulphate entirely eliminated the first decline. The final fall of the curves was caused by a decrease in the sugar concentration of the medium.

With molasses obtained from other factories the time of appearance and the size of the declines were variable. However, the minima of declines always appeared at the same intervals. Figs. 3 and 4 show the results obtained with molasses produced by the Szerencs and Selyp sugar factories, respectively.

The fermentation rates in molasses samples containing identical concentrations of sugar, in molasses supplemented with ammonium sulphate and in pure sucrose solution, were also compared. In pure sucrose fermentation ceased after a sudden initial increase (Fig. 5). In this solution the intensity of fermentation was very low. Therefore in subsequent experiments, in order to decrease the error of measurement, the yeast concentration was increased to 0.4 g/100 ml buffer.

The intensity of fermentation in sucrose supplemented with different amounts of ammonium sulphate is presented in Fig. 6. The results uniformly indicated that, probably owing to the lack of bios factors, the fermentation rate in sucrose is not increased by the addition of nitrogen.

Finally the effect of temperature on the appearance of the declines was examined. As expected, fermentation intensity increased with rising temperatures and the declines appeared more rapidly (Fig. 7). In order to increase the accuracy of measurement, in these experiments 0.25 g yeast was suspended in 100 ml buffer. Due to the higher yeast concentration, the sugar content of the medium was exhausted more rapidly and thus only the first decline could be measured. Therefore in these experiments the fermentation rate was estimated only within 4 hours.

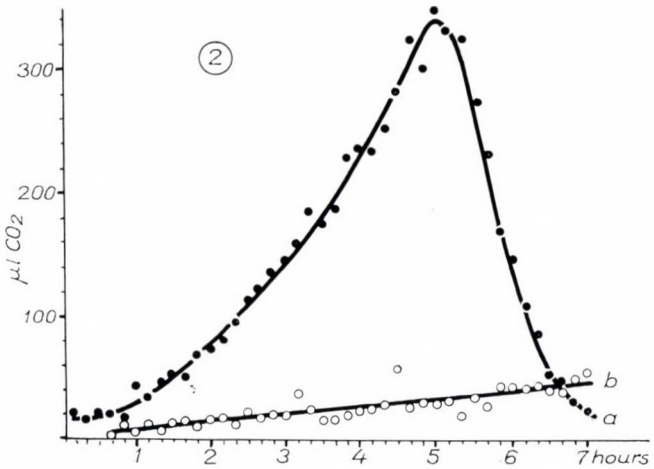

Fig. 2. Fermentation (a) and respiration (b) of synchronously budding yeast culture in ammonium sulphate supplemented molasses-phosphate medium. Yeast concentration, 0.1 g/100 ml buffer

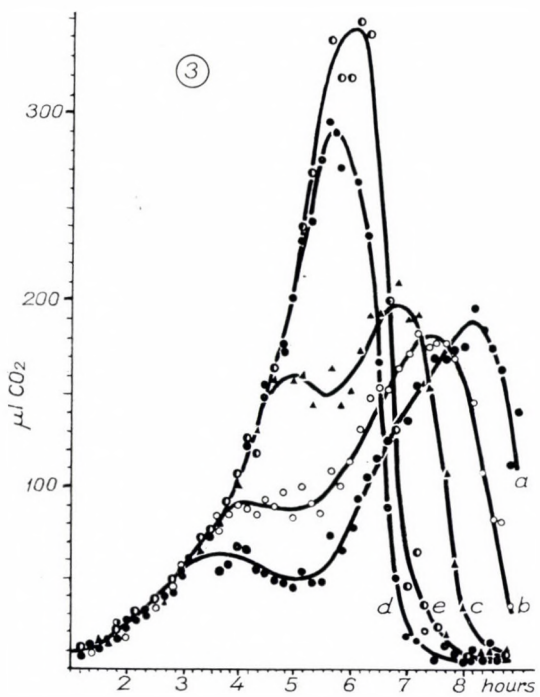

Fig. 3. Influence of nitrogen source content on the rate of fermentation. Molasses (Szerencs Sugar Factory) medium supplemented with phosphate buffer containing 0 (curve a), 0.025 (curve b), 0.05 (curve c), 0.1 (curve d), and 0.2 (curve e) per cent ammonium sulphate. Yeast concentration, 0.1 g/100 ml buffer

Discussion

The present investigations have shown that in synchronously dividing yeast cultures the increase in fermentation rate ceases at certain intervals and even the absolute intensity of fermentation may decrease. Such declines can well be observed in the graph expressing fermentation vs. time. At the intervals corresponding to the declines, about 70 per cent of the cells carried

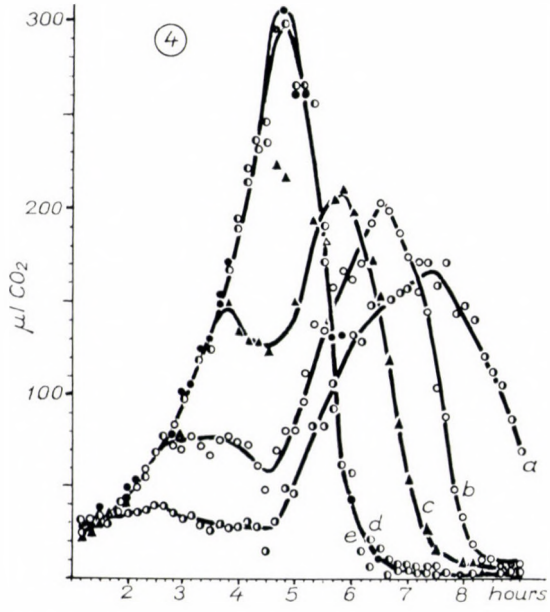

Fig. 4. Influence of nitrogen source content on the rate of fermentation. Molasses (Selyp Sugar Factory) medium supplemented with phosphate buffer containing 0 (curve a), 0.025 (curve b), 0.05 (curve c), 0.1 (curve d), and 0.2 (curve e) per cent ammonium sulphate. Yeast concentration, 0.1 g/100 ml buffer

buds. This finding indicates an association between the occurrence of declines and the mitotic cycle of cells. This consideration has been supported by the fact that in the fermentation curve for the seed yeast culture, which is richer in nitrogen than baker's yeast, the first decline is missing and the second is less definite.

The influence of incubation temperature on the appearance of declines is clearly associated with the effect of higher temperatures on generation time. The low fermentative activity of the used yeast in pure sucrose was attributable to the absence of bios factors, since it remained unaffected by the addition of ammonium sulphate.

The conclusion has been drawn that the yeast is able to decompose its own carbohydrate-splitting enzymes when the nitrogen required for multi-

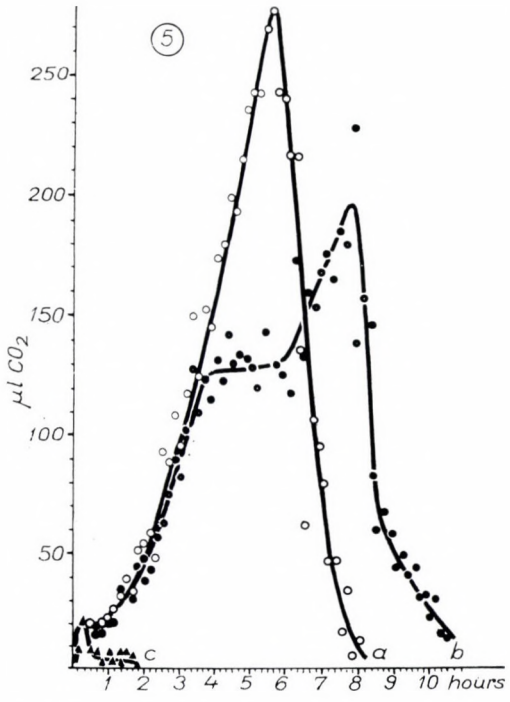

Fig. 5. Fermentation in different media. Curve a: molasses + phosphate buffer + ammonium sulphate; Curve b: molasses + phosphate buffer; Curve c: sucrose + phosphate buffer. Yeast concentration, 0.13 g/100 ml buffer

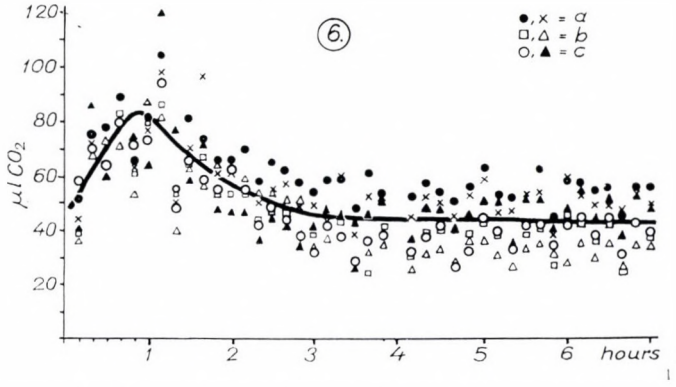

Fig. 6. Fermentation in sucrose medium supplemented with phosphate buffer containing a = 0.05, b = 0.1, c = 0.2 per cent ammonium sulphate. The points obtained in 2 parallel experiments are shown separately. Yeast concentration, 0.4 g/100 ml buffer

plication is not supplied in the medium. However, under the same conditions members of the respiratory enzyme system are not attacked. This finding may be explained by the fact that carbohydrate-splitting enzymes occur dissolved in the cytoplasm and are easily attacked by proteolytic enzymes. In contrast members of the respiratory enzyme system are present in the

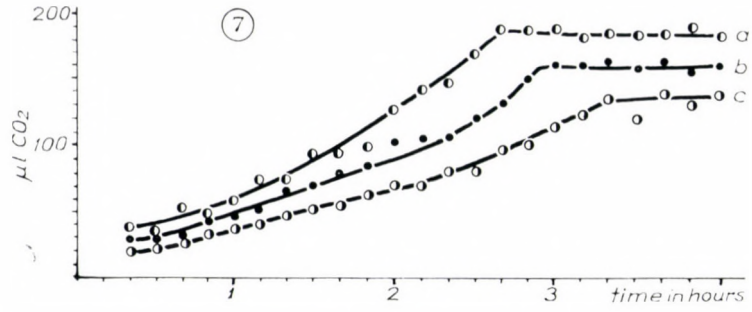

Fig. 7. Fermentation by synchronously budding yeast in molasses phosphate medium at 32° (a), 30° (b), and 29° (c) C temperatures. Yeast concentration, 0.25 g/100 ml buffer

mitochondria, and thus, provided the mitochondrial structure is intact, are inaccessible for proteolytic enzymes. To confirm the validity of these considerations further experiments are needed.

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STUDIES ON L FORMS OF STAPHYLOCOCCUS AUREUS STRAINS OF DIFFERENT ANTIBIOTIC AND PHAGE SENSITIVITY

By

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Summary. (i) The production of L forms by *Staphylococcus aureus* in the presence of 20 μg/ml Celbenin is associated with antibiotic sensitivity. As compared to sensitive strains, antibiotic resistant cultures produced L forms less frequently.

(ii) Strains belonging to phage types 80/81 and 52/52A/80/81 are less liable to L transformation.

(iii) Eight out of 10 staphylococcal L strains grew readily and formed mucous deposit in a liquid medium containing 5 per cent sucrose, 10 per cent human serum and 3 per cent NaCl.

(iv) When applied at concentrations of 0.3 per cent or higher, alpha or gamma globulin and albumin serum fractions could be substituted for the human serum.

(v) Of 8 L strains growing readily in the presence of serum, 2 were able to multiply in a semisynthetic medium containing 0.3 per cent Proteose peptone (Difco). Maximum growth was reached in 18—24 hours. Reversion did not occur when Celbenin was omitted from the medium.

(vi) A considerable morphological difference was observed between the serum and Proteose peptone cultures of the same organism.

In recent year several investigators have studied the transformability to L forms of *Staphylococcus aureus*. The experiments yielded variable results. According to SCHÖNFELD [1] all freshly isolated staphylococcal strains could be transformed. PROZOROVSKI [2] demonstrated that every antibiotic sensitive strain produced L forms. In contrast, WILLIAMS [3] obtained only 4 L strains out of 25 cultures.

In the present study the association between the production of L forms and the biological properties of *Staph. aureus* strains was examined. The investigations were extended to the cultural and morphological properties of L forms in order to obtain stable L forms growing readily in liquid medium.

Materials and methods

Culture media. Medium 1 contained: beef extract, 3.7 per cent; $Na_2HPO_4 \cdot 7H_2O$, 2.0 per cent; NaCl 3.0 per cent; agar, 1.0 per cent; human serum 10.0 per cent in distilled water; and was adjusted to pH 7.3.

Medium 2 contained: beef extract, 3.7 per cent; $Na_2HPO_4 \cdot 7\ H_2O$, 2.0 per cent; NaCl, 3.0 per cent; sucrose, 5.0 per cent; human serum, 10.0 per cent in distilled water; and was adjusted to pH 7.3.

Medium 3 contained: casein hydrolysate (Difco), 1.0 per cent; NaCl, 3.5 per cent; $Na_2HPO_4 \cdot 7\ H_2O$, 1.25 per cent; KH_2PO_4, 0.05 per cent; sucrose, 5.0 per cent; $MgSO_4$, 0.001 per cent; $MnSO_4$, 0.001 per cent in distilled water; and was adjusted to pH 7.4.

Medium 4 contained: casein hydrolysate (Difco) 1.0 per cent; NaCl, 3.5 per cent; $Na_2HPO_4 \cdot 7\ H_2O$, 1.25 per cent; KH_2PO_4, 0.05 per cent; sucrose, 5.0 per cent; $MgSO_4$, 0.001

per cent; $MnSO_4$, 0.001 per cent; agar (Noble), 1.0 per cent in distilled water; and was adjusted to pH 7.4.

The media were autoclaved for 1 hour at 121° C; after sedimentation the clear medium was reautoclaved for 20 minutes at 121° C.

For the cultivation of unstable L forms 20 μg/ml Celbenin was added to the medium.

Estimation of antibiotic sensitivity was performed by the method of László *et al.* [24]. Agar plates were seeded with 1 ml 1 : 10 diluted 20 hour broth culture. The plates were then left to stand at room temperature for 90 minutes. Schleicher and Schuell No 2043/B paper discs were then placed onto the plates. The discs contained 50 μg penicillin, streptomycin, terramycin, or aureomycin, 30 μg chloramphenicol or erythromycin. Cultures with inhibition zones larger than 25 mm were considered sensitive, those with inhibition zones smaller than 8 mm were considered resistant to the corresponding antibiotic.

Disintegration of bacteria was performed in a M. S. E. ultrasonic disintegrator. Five ml samples of staphylococcal L forms were treated for 20 seconds at 1.7 ampere intensity.

Fractionation of human serum was carried out by means of a DEAE cellulose column as described by Sober and Peterson [25].

The fractions were dialysed against saline at 4° C for 24 hours. Protein content was determined at 280 mμ by use of the Juan spectrophotometer and a standard curve.

Microphotographs were taken partly with the phase contrast system of the Lumipan (Zeiss, Jena) microscope, partly by use of the 3D condenser at dark-field illumination.

Results

Production of L forms on solid medium. Plates of medium 1 were seeded with 0.1 ml samples of the 14-hour broth cultures of 108 different *Staph. aureus* strains. The Celbenin-containing medium 1 was a modification of the media devised by Dienes and Sharp [4] and Schönfeld [1]. Out of the 108 strains 50 produced L colonies. In each colony a central part growing into the medium and a typical surface growth consisting of large bodies were distinguished (Fig. 1).

As seen in Table I, II, and III, the ability to produce L forms is associated with antibiotic sensitivity and phage type. Strains sensitive to antibiotics produced L colonies with 69, those resistant to 1 or 2 antibiotics with 45, and

Table I

Transformability to L forms of Staphylococcus aureus strains of different antibiotic sensitivity

Antibiotic pattern	No. of strains	Percentage of transformable strains
Sensitive	13	69
P. res.-sensitive.....	66	45
Resistant	29	37

Sensitive: sensitive to penicillin, streptomycin, chloramphenicol, terramycin, aureomycin, erythtromycin

P. res.-sensitive: Resistant to penicillin, sensitive to streptomycin, chloramphenicol, terramycin, aureomycin, erythromycin

Resistant: resistant to 3 or 4 of the above antibiotics

those resistant to 3 or 4 antibiotics with 37 per cent frequency. Table II shows that strains not capable of producing L forms occurred mainly in phage group I. From Table III it is evident that within phage group I types 80/81 and 52/52A/80/81 produced L forms less frequently (only 2 out of 23 strains).

Table II

Transformability to L forms of Staphylococcus aureus strains of different phage groups

Phage group	No. of strains	No. of transformable strains
I	41	14
II	10	7
III	28	13
IV	2	1
Mixed	14	6
Untypable	6	3
756/950	7	5

Table III

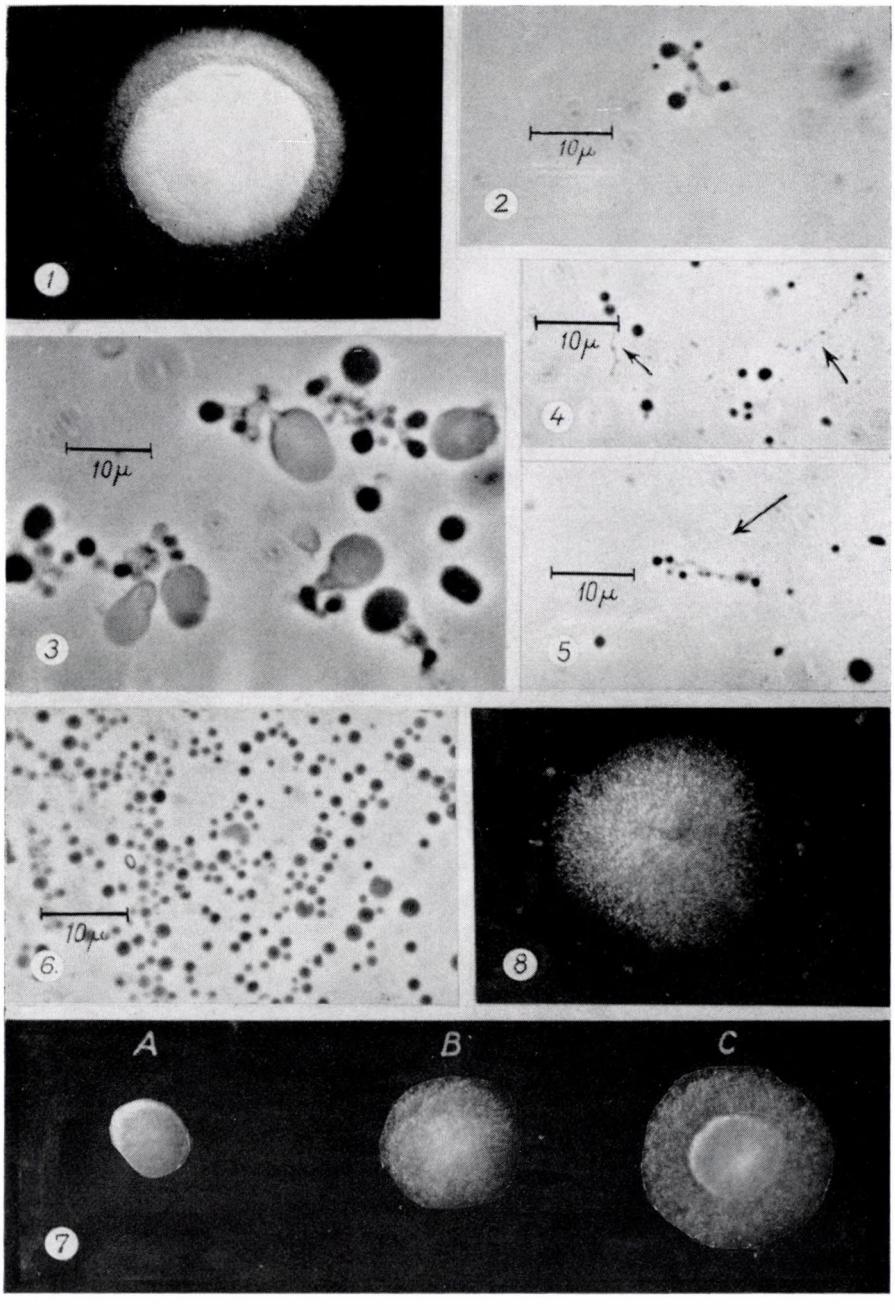
Transformability to L forms of Staphylococcus aureus strains belonging to phage group I

Phage type	No. of strains	No. of transformable strains
80/81	17	2
79	9	5
52/52A/80/81	6	0
29	7	5
29/52	2	2

All strains included in Table III were resistant to penicillin and sensitive to streptomycin, chloramphenicol, terramycin, aureomycin, erythromycin.

Colonies visible to the naked eye appeared with 2 strains after 48 hours, with the rest after 96 to 120 hours.

Cultivation of L forms in liquid serum medium. Of L strains derived from staphylococci of different antibiotic sensitivity and phage type, 10 cultures producing large colonies on medium 1 were selected. From plate cultures on medium 1 of these strains blocks were cut and transplanted to medium 2. To medium 2 sucrose was added at 5 per cent concentration, as in preliminary


1
2
10μ
3
10μ
4
10μ
5
10μ
6
10μ
8
A
B
C
7

examinations this substance promoted the growth of L forms. Of the 10 strains 8 grew in the liquid medium after 1 or 2 weeks incubation at 37° C. In further subcultures the rapidity of growth gradually increased. After the 4th or 5th subculture maximum growth was obtained within 48 hours. Growth in liquid always occurred as a mucous sediment.

Ultrasonic disintegration for 20 seconds in the M. S. E. apparatus did not influence the viability of the cultures. Further transfers were performed with cultures treated in this manner, as ultrasonic disintegration destroyed elements larger than 1 μ. Thus the development of the L organism, appearance of forms different size and production of large bodies, could be observed. Figures 2 and 3 show the 12 and 24 hour cultures of *Staph. aureus* strain 100 in medium 2, with dividing forms measuring 1 μ. Later large bodies of different size multiplying in clumps appeared. In ageing cultures the size of these elements gradually increased. After 48 hours a gradual rupture of the large bodies was observed and the culture contained more and more debris.

The transfer of liquid medium cultures was attempted in Celbenin-free medium. In the absence of this antibiotic L strains reverted rapidly in 4 to 14 days into cocci. Neither of the L strains could be stabilized for 20 passages.

Role of serum. The above L cultures formed mucous deposit in human serum-containing liquid medium. WEIBULL and LUNDIN [5] mentioned that omission of serum eliminated this type of growth. In our experiments the cultures failed to grow without serum. Assuming that a specific factor was required for growth, the role of serum fractions was investigated. Human serum was run through DEAE cellulose column and the obtained fractions were added to medium 2. It was revealed that except for the beta fraction, growth occurred in the presence of 0.3 per cent or higher concentration of all fractions.

Effect of Proteose peptone. As the growth in the presence of serum was not due to one special serum factor, a synthetic medium, in which the parent strains grew readily, was composed (medium 3). However, all L forms failed to grow in this medium. The basal medium was then completed with various peptones, and seeded with large inocula. L strains 100 and 53/101 started

Fig. 1. L colony of *Staph. aureus* strain 100 on medium 1 containing 20 μg/ml Celbenin, after 48 hours. Dark-field micrograph, × 126

Fig. 2. L form of *Staph. aureus* strain 100 grown in medium 2 for 12 hours. Inoculation was performed with disintegrated culture. Phase contrast micrograph, × 1134

Fig. 3. L form of *Staph. aureus* strain 100 grown in medium 2 for 24 hours. Inoculation was performed with disintegrated culture. Phase contrast micrograph, × 1134

Figs. 4, 5. Stable L form of *Staph. aureus* strain 100 grown in medium 3 for 16 hours. Phase contrast micrograph, × 1134

Fig. 6. Stable L form of *Staph. aureus* strain 100 grown in medium 3 for 48 hours. Phase contrast micrograph, × 1134

Fig. 7. The three colonial types of stable L form of *Staph. aureus* strain 48 on medium 1. Dark-field micrograph, × 126

Fig. 8. Surface colonies of stable L form of *Staph. aureus* strain 100 on medium 4; × 126

to grow abundantly after 5 days at 37° C in the medium containing 0.3 per cent Proteose peptone (Difco). Other strains did not grow appreciably after a 4 week incubation period. Subcultures of the two former strains exhibited maximum growth within 18 to 24 hours.

In subsequent experiments the range of peptone concentration permitting growth was determined. It was found that at least 0.04 per cent peptone was required for growth. Optimal growth was obtained at 0.3 per cent concentration.

Morphological examination of L forms growing in serum-free medium. The two strains growing readily both in Proteose peptone-supplemented medium 3 and in medium 2 were studied. Maximum growth was reached in 18 hours in medium 3 and in 48 hours in medium 2. The two different media yielded cultures of very different morphological appearance. In the presence of serum 24 hour cultures consisted of large bodies occurring in clumps. In contrast, the semi-synthetic, Proteose peptone-supplemented medium yielded after 16 hours a mass of filaments consisting of particles smaller than 1 μ. Morphologically these elements resembled PPLO cultures (Figs. 4 and 5). The smallest granules within the chains measured about 0.3 μ. In 48-hour cultures the filaments disappeared and gave place to large bodies, which, however, never reached the 5—15 μ size observed in serum-containing medium (Fig. 6). Strains 100 and 53/101 were similar in morphology. The slight differences between the two cultures were as follows. (1) In the early stage of multiplication strain 53/101 produced less filaments than strain 100 did. (2) Strain 53/101 produced somewhat larger bodies than those formed by strain 100.

In shake cultures seeded with large inocula an increase in turbidity was observed after 18—24 hours. In such cultures filaments were not formed and single or double L bodies predominated. The size of these elements varied between less than 1 and 3 μ. When the cultures were transferred to medium 4 for determining viable counts, the shake culture yielded a considerably smaller number of colonies than the culture incubated without shaking. This observation and the finding that for obtaining a good growth, shake cultures should be inoculated heavily, indicate that shaking is unfavourable for the cultivation of these L forms.

The behaviour of the cultures on solid media 1 and 4 was interesting. As shown in Fig. 7, on medium 1 three types of colonies can be distinguished. Type A grows into the agar, type B forms colonies on the surface and type C grows partly in the agar, partly on the surface. In contrast, on medium 4 only surface colonies occurred (Fig. 8). The colonies appeared on medium 1 after 48—72 hours, in medium 4 after 24 hours.

Stability of L forms im medium 3. In Celbenin-free Proteose peptone medium 2 strains 100 and 53/101 were transferred more than 50 times without alteration.

In order to exclude errors due to contamination, the coagulase reaction of the strains was checked. Both cultures retained their ability to coagulate citrated human plasma.

Therefore it was considered that both strains yielding rapid and abundant growth in Proteose peptone medium were stable L forms and originated undoubtedly from *Staph. aureus* strains 100 and 53/101.

Discussion

Transformation of Gram positive cocci into L forms has been observed to occur only on solid medium supplemented with serum and an adequate amount of salt [4]. In experiments performed by DIENES and WEINBERGER [6], MINCK [7], MINCK and LAVILLAUREIX [8] and EDWARD [9], horse serum, human serum, or human ascitic fluid gave the most satisfactory results. WEINBERGER [10] isolated L forms from 46 per cent of *Staph. aureus* strains on solid medium containing NaCl, human serum and 20 μg/ml Celbenin. SCHÖNFELD [1] and PROZOROVSKI [2] obtained a higher, while WILLIAMS [3] a lower frequency of successful isolation of L forms.

The transition of antibiotic resistant strains into L forms is less likely than that of sensitive cultures. In experiments not shown in the Tables, of 50 *Staph. aureus* strains resistant to 5—6 antibiotics only 6 per cent could be transformed. VÁCZI *et al.* [11] observed that polyresistant strains contain lipid substances in the outer layers of the cell wall. These substances protect the cell against phagocytosis, and their presence indicates a close connexion between the protoplasmic membrane and the cell wall. This close connection may explain the difficult transformability of polyresistant strains to L forms.

It is known that phage types 80/81 and 52/52A/80/81 represent a separate pathogenetic unit. These staphylococci are uniform in the degree of hyaluronidase, phosphatase and coagulase production [12—14]. The present experiments confirmed the uniform nature of these strains, as in contrast to other types of phage group I, the transformation of these strains to L forms was difficult. Since the examined strains were resistant to penicillin but sensitive to other antibiotics, this finding cannot be explained by the antibiotic resistance of the cultures. Other phage types with similar sensitivity pattern were transformed in 45 per cent as opposed to the 8 per cent isolation of L forms from 80/81 strains. This observation also indicates that there is a difference in the surface structure of the two kinds of cultures.

Some L forms grew readily in liquid medium containing 5 per cent sucrose. Cultures developing after ultrasonic disintegration resembled morphologically unstable L forms of other bacteria. Short ultrasonic disintegration did not affect the viability of *Staph. aureus* L forms. PANOS *et al.* [15] made similar observations for streptococcal L forms.

Two different theories have been advanced as to the role of serum. According to ALTENBERN [16], WEIBULL and LUNDIN [5], MEDILL and O'KANE [17], LORKIEWITZ [18] and MATTMAN *et al.* [19], serum acts as a detoxifying factor. On the other hand, in PPLO strains a specific effect is attributed to the lipoprotein fraction of serum. SMITH *et al.* [20] and RODWELL [21] regard serum as a source of atoxic unsaturated fatty acids. The present experiments have demonstrated that serum plays no specific part in the multiplication of staphylococcal L forms.

A morphological difference has been shown between the serum-meat extract and Proteose peptone medium cultures of the same L forms. Apart from differences manifesting themselves in filamentous (PPLO-type) and clumpy growth, differences in the size of L forms were also observed. The size of the smallest reproducible element in serum-containing medium 1 was about 1 μ, while of that in medium 3 was about 0.3 μ. The use of different media may explain the discrepancy between the results of WEIBULL [22] and WILLIAMS [3] regarding the size of the smallest reproductible staphylococcal L form units (0.3 and 0.7 μ, respectively). The morphological difference is evident also on solid medium, as on serum-free medium surface colonies not growing into the agar predominate. A similar observation was made for Proteus L forms by WEIBULL [5].

The criteria for the stability of *Staph. aureus* L forms have been discussed by several authors. PROZOROVSKI [23] considers the L forms stable if they are unable to revert through serial passages in penicillin-free medium. In contrast, according to SCHÖNFELD, even strains subcultured many hundred times without penicillin cannot be considered stable, as reversion of such cultures can be induced by altering the medium.

In our opinion PROZOROVSKI's criterium of stability is justified. It would therefore appear that our serum-free medium is suitable for the isolation of stable *Staph. aureus* L forms and for the subcultivation of these stable strains.

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ISOLATION AND CHARACTERIZATION OF SOME B. SUBTILIS PHAGES WITH "TRANSFORMING" ACTIVITY

By

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Summary. In order to obtain nucleic acid preparations with transforming activity, a number of *B. subtilis* phages were isolated. Some characters of the isolated phages such as host-range, adsorbing capacity, latent period and burst-size were determined. The phages were grouped on the basis of serological properties and were compared with phages recently isolated by other authors. A close serological relationship was revealed among agents that seemingly corresponded to temperate phages. The nucleic acid yielded by virulent phages exerted a transforming activity on competent cells of *B. subtilis*.

Phages acting on *B. subtilis* have been studied extensively. Experiments were carried out as to the antigenic structure [1], morphology [2], X-ray [3] and visible light [4] sensitivity of these agents. In recent years new subtilis phages have been isolated and more precisely described. ROMIG and BRODETSKY [5] characterized 6 of several subtilis phages. THORNE [6] and TAKAHASHI [7] described a phage with transducing activity. IVÁNOVICS and CSISZÁR [8] isolated a major group of transducing phages. The purpose of the present work was to obtain new subtilis phages and to use them for the preparation of isolated and purified nucleic acids exerting transforming activity on bacterial cells.

Materials and methods

Cultures. B. subtilis 168 I$^-$ auxotrophic and prototrophic strains used for the isolation and propagation of phages were obtained from the laboratory of *Dr.* W. SZYBALSZKI. For the determination of host range 4 auxotrophic cultures obtained from *Dr.* W. SZYBALSZKI, 2 prototrophic cultures (TAKAHASHI's strain SB19 and THORNE's strain W23) and 4 additional *B. subtilis* cultures of our own collection were used. Among other bacteria employed in host-range experiments the following strains were included: *B. megaterium* KM, 507 and 207, *B. anthracis* A76 and A87, *B. cereus* C114, C116, C569, C4 and *B. licheniformis* NRS821 and NRS1108.

Phages. The isolated phages were compared with *E. coli* phages T_1, T_2, T_4 and T_6 and with THORNE's phage SP10 and TAKAHASHI's phage PBS1.

Media. For phage titration YP medium [9] was used. The solid medium contained 1.5 per cent, the soft medium 0.75 per cent agar. Cells infected with phage DNA were incubated on YT medium containing 1 per cent Tryptone (Difco), 0.5 per cent YE (Difco) and 0.5 per cent NaCl. In transduction experiments the GGM medium described by IVÁNOVICS and CSISZÁR [8] was employed. For the development of competence the cultures were grown in the synthetic liquid medium of ANAGNOSTOPOULOS and SPIZIZEN [10].

* Fellow of the *A. von Humboldt Foundation (Bad Godesberg bei Bonn)* in the years 1962—63.

Isolation of phages was performed by the method of ADAMS [11]. The phages occurring in 6 different soil samples were simultaneously propagated on auxotrophic and prototrophic *B. subtilis* 168 I^- cells. Then plates were poured with dilutions of the phage suspensions and the resulting plaques were transferred to the culture of the corresponding bacterium. Homogeneity of the phages was ensured by several subcultures. The lysates were treated with chloroform, stabilized with 0.5 per cent gelatin, and stored in the refrigerator. Counting of plaque-producing units, determination of latent period and burst-size was performed by the standard methods of ADAMS [11]. Transduction was carried out as described by IVÁNOVICS and CSISZÁR [8].

Antibodies. Rabbits were given for a 4 week period two intravenous injections weekly of the lysate concentrated by centrifugation.

Isolation of phage DNA was based on the method described by PARANCHYCH [12]. The phage concentrated by centrifugation was dissolved in a mixture containing phosphate buffer, sodium lauryl sulphate and ethylenediaminotetraacetate (EDTA). Proteins were removed by shaking out the mixture three times with phenol. Finally DNA was precipitated with two volumes of ethanol. The precipitate was dissolved in 5 *mM* EDTA. The purity of DNA precipitate was checked by classical microanalytical methods and spectrophotometry.

Competent cells were developed as described by ANAGNOSTOPOULOS and SPIZIZEN [10].

Infection of competent B. subtilis cells was performed by adding 0.5 ml bacterial suspension (optical density, 0.1) to 0.5 ml DNA dilution (10 to 1 μg/ml) and incubating the system in a 30° C water bath for 25 minutes. After the addition of 1 ml soft agar, the mixture was spread over the surface of a tryptone agar plate. Further incubation was carried out at 37° C for 16 hours. For details, see the paper of FÖLDES and TRAUTNER [13].

Results

By use of the auxotrophic strain of *B. subtilis* 168 I^-, 23 seemingly different phages were isolated. The prototrophic strain yielded 10 phages. On the basis of plaque morphology 3 main types were distinguished (Figs. 1—3). The majority of phages isolated with the aid of the auxotrophic strain (SP50—SP90), produced clear or target-like plaques, while phages obtained with the prototrophic strain, apart from some exceptions, formed turbid plaques. The morphology of plaques was highly influenced by the ingredients of the medium, the temperature, and the humidity at incubation. Our observations in this respect were in accordance with those of ROMIG and BRODETSKY [5].

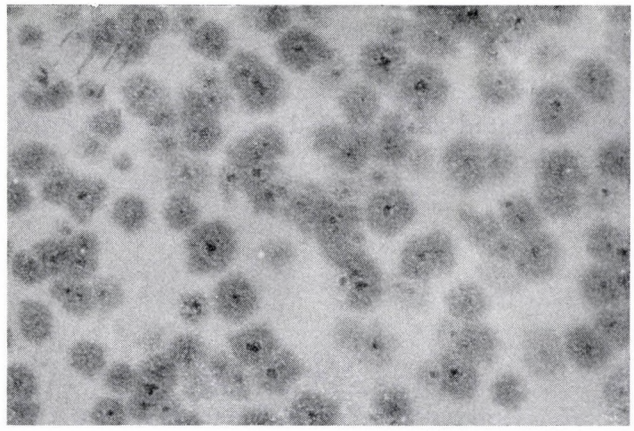

Fig. 1. Turbid plaques. Magnification 1/2

The plaque morphology was insufficient for the characterization of the isolated phages. An adequate grouping was achieved by considering the antigenic properties of the phages. By use of rabbit sera prepared against a virulent (SP50) and against a seemingly temperate (SP100) phage, 3 groups

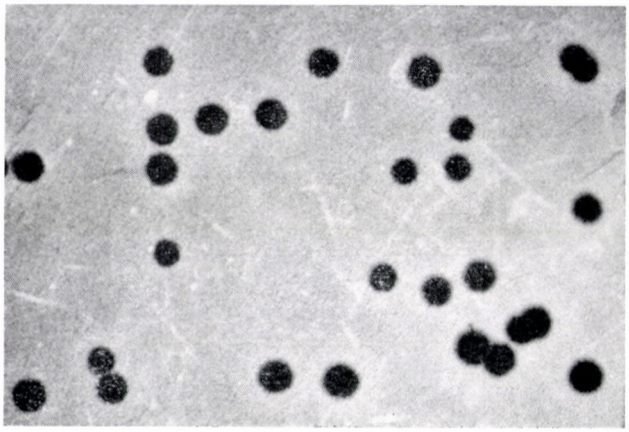

Fig. 2. Clear plaques. Magnification 1/2

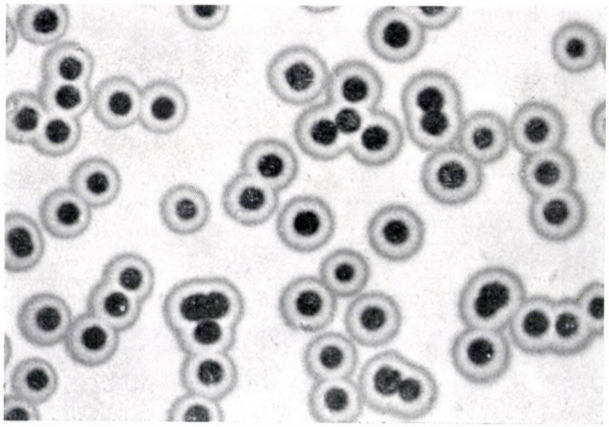

Fig. 3. Target-like plaques. Magnification 1/2

were distinguished on the basis of major differences. By taking into account minor differences, the phages could be divided into 6 groups. Table I shows the result of phage neutralization (K values), which served as a base for grouping.

For examining other characters of the isolated phages, two members of each serologically uniform group were selected. The results are presented in Table II.

Table I

Grouping of the isolated phages on the basis of K values obtained with two different sera

Phages	Plaque	Antibodies		Phages	Plaque	Antibodies		Phages	Plaque	Antibodies	
		SP50	SP100			SP50	SP100			SP50	SP100
		K values				K values				K values	
SP50	cl	200	0	SP80	t	105	420	SP90	t	0	220
SP51	cl	175	0	SP81	t	60	460	SP91	cl	0	200
				SP82	cl	50	400	SP92	cl	0	50
SP60	cl	75	0	SP83	t	50	400	SP93	cl	0	50
SP61	cl	45	0	SP84	t	45	480				
SP62	cl	120	0	SP85	t	40	420	SP100	t	0	345
								SP101	t	0	460
SP70	tl	0	0					SP102	t	0	400
SP71	cl	0	0					SP103	t	0	400
SP72	cl	0	0					SP104	t	0	350
SP73	cl	0	0					SP105	t	0	430
SP74	cl	0	0					SP106	t	0	500
SP75	cl	0	0					SP107	t	0	600
SP76	cl	0	0					SP108	t	0	300
SP77	cl	0	0					SP109	t	0	430

Abbreviations: cl = clear plaque; t = turbid plaque; tl = target-like plaque.

Table II

Specificity of the studied B. subtilis phages on the basis of host-range experiments

Phages 10^7/ml	*B. subtilis* 12 strains	*B. licheniformis*		*B. mycoides*	*B. pumilus* NRS736	*B. cereus* C569, C4, C114, C116	*B. megaterium* KM, 507, 207	*B. anthracis* A76, A87
		NRS821	NRS1108					
SP50	+ cl	+ cl	—	—	—	—	—	—
SP51	+ cl	+ cl	+ t	—	—	—	—	—
SP60	+ cl	+ cl	+ t	—	—	—	—	—
SP61	+ cl	+ cl	+ t	—	—	—	—	—
SP70	+ tl	—	—	—	—	—	—	—
SP76	+ cl	+ cl	+ t	—	—	—	—	—
SP80	+ t	+ cl	+ cl	—	—	—	—	—
SP82	+ cl	+ cl	+ t	—	—	—	—	—
SP90	+ cl	+ cl	+ t	—	—	—	—	—
SP100	+ t	+ cl	+ t	—	—	—	—	—
SP101	+ t	+ cl	+ t	—	—	—	—	—

Abbreviations: + = phage sensitivity; cl = clear plaque; tl = target-like plaque; t = turbid plaque.

From Table II it is clear that the isolated phages were highly specific. All *B. subtilis* strains were sensitive, while among other cultures belonging to the *Bacillus genus* only *B. licheniformis* showed some susceptibility. Some other characteristic properties of the selected phages are shown in Table III.

Table III

Some characters of the examined B. subtilis phages

Phage	Plaque	Adsorption % after min.		Burst size	Latent period minutes
		1/2	12		
SP50	cl	96	97	200	60
SP51	cl	89	92.5	200	60
SP60	cl	92	92.5	200	60
SP61	cl	50	87	100	60
SP62	cl	34.5	75.5	200	60
SP70	tl	97	97.5	100	55
SP75	cl	20	70	100	60
SP80	t	94.6	96.5	50	55
SP90	t	83.5	91	50	55
SP100	t	95	97	25	55
SP101	t	84.5	97	40	55

Abbreviations: cl = clear plaque; tl = target-like plaque; t = turbid plaque.

The data indicated that (a) the maximum adsorption of some *B. subtilis* phages occurred within one half minute, of others only after 12 minutes; (b) the latent period lasted 55 or 60 minutes; (c) the number of phages liberated from one cell varied between 25 and 200.

The curve representing the result of the one step multiplication experiment was usually uniform for all phages. Therefore only the curve for SP50 is presented (Fig. 4).

According to the studies of WOLLMANN and LACASAGNE [3] and GIUNTINI *et al.* [2] *B. subtilis* phages are 80×120 and 80×200 mμ in size, respectively, and therefore they belong to the group of large phages. As to our phages the same conclusions can be drawn. An electronmicrograph taken by KELLENBERGER and BOY DE LA TOUR [13] revealed that phage SP50 measured $80 \times 80 \times 170$ mμ in size. Our phages, as shown by centrifugation experiments, were of the same order. When a Seitz filter was washed through with broth, it retained 95—99 per cent of the subsequently filtered phages. All phages could be centrifuged with the Janetzky Model T 14-R centrifuge operating at

21 000 *g*. Comparative studies showed that in 1 1/2 hours sedimentation occurred with T_1 phages in 94, with T_4 phages in 99.4, with T_6 phages in 99.6, and with phage SP50 in 98 per cent. Thus, as regards sedimentation, phage SP50 occupies an intermediary position between phages T_1 and T_4.

B. subtilis phages can be precipitated with ammonium sulphate without an appreciable loss of activity. Increasing concentrations of ammonium sul-

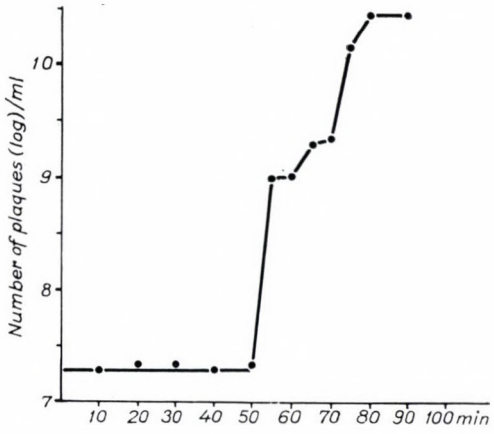

Fig. 4. One step growth of *B. subtilis* bacteriophage SP50

phate precipitate increasing amounts of phages. At 50 per cent saturation 99 per cent of the phage particles is precipitated. Higher concentrations of this salt do not increase further the amount of precipitated phages.

Table IV

Comparison of B. subtilis phages of different origin

Phage	Author	Antibodies (K values)		Transduction	Infectivity of DNA
		SP50	SP100		
SP50	Földes	200	0	—	+
SP100		0	460	—	—
SP10	Thorne	0	435	+	
PBS1	Takahashi	0	286	+	

The DNA content of our *B. subtilis* phages could easily be isolated. The isolated and purified DNA was suitable for infecting competent cells. Quantitative experimental results indicated that successful infection, in addition to competency, depended on the ingredients of the media, temperature

and other factors. The factors influencing the mechanism and kinetics of infection have been dealt with in other papers [13, 14].

While the isolated and purified DNA is suitable for "transformation" the original phages, as compared to the transducing phages of IVÁNOVICS and CSISZÁR, exert no transducing activity.

Our phages, which had been isolated by use of SZYBALSZKI's auxotrophic and prototrophic strains, differed in several respects from other subtilis phages described recently. The transducing phage of THORNE [6] was isolated with *B. subtilis* strain W23 and could be propagated on *B. licheniformis*. TAKAHASHI [7] used strain SB19 for the isolation and propagation of phage PBS1. For the same purpose IVÁNOVICS and CSISZÁR [8] used *B. subtilis* strain Marburg.

The phages of different origin could be also differentiated on the basis of the transducing and transforming ability and their serological properties (Table IV).

Experimental results set in Table IV indicate that phage SP50 comprises a separate serological group and that phage SP100 is antigenically related to the phages of THORNE and TAKAHASHI. The antigenic identity of phages SP100 and SP10 is evident; however, phage PBS1 somewhat differs in serological behaviour. Transduction experiments with SP50 and SP100, using phages SP10, PBS1 and 3NT as controls, were unsuccessful. No transformation experiments were carried out with DNA of the control phages. Successful transformations were obtained with DNA prepared from SP50, whereas under similar conditions SP100 DNA failed to act as a transforming agent.

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STUDIES ON THE HUMAN INTESTINAL FLORA

I. THE NORMAL INTESTINAL FLORA AND THE STABILITY OF ITS CONSTITUENTS

By

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Summary. (i) *E. coli*, *Str. faecalis*, *L. bifidus* and Bacteroides have been found to comprise the 4 constant members of the faecal flora. Accidentally encountered microorganisms were in the order of frequency: clostridia, *Kl. pneumoniae*, staphylococci, Proteae, microaerophilic lactobacilli, saccharomycetes, aerobic spore-bearing bacilli and *Ps. aeruginosa*.

(ii) The most frequent member of the constant flora was *E. coli*, *L. bifidus*, Bacteroides and *Str. faecalis* were next in order. The high *E. coli* count, the significantly higher standard error and higher deviations at increased bacterial counts in the distributions of aerobic constituents were attributable to the fact that the examined material originated from older bedridden patients with frequent disorders of the intestinal passage. The older age group differed from the younger only in increased *E. coli* counts, which caused a shift in the distribution of organisms constituting the intestinal flora.

(iii) The number and distribution of permanent constituents showed a great individual variation. In contrast, the bacterial count in the same person was always constant.

(iv) A restitution in the number and proportion of permanent bacteria occurred within a short period (1 week), even when the normal equilibrium had been seriously upset. In the regeneration process the original predominant *E. coli* strain tended to reappear.

(v) In view of the deviations in the constant and accidental constituents of the intestinal flora, eubiotic and dysbiotic conditions should be judged cautiously. The occurrence of damages in the intestinal flora can properly be estimated only in the possession of quantitative data on the preceding normal conditions.

The exact knowledge of the biological and pathogenetical significance of the human intestinal flora has become of decisive importance since the introduction of antibiotic therapy. If antibiotic treatment causes the destruction of the normal bacterial flora in addition to that of the pathogenic agent, it is obvious that the decrease in the number of normal or an increase in the number of certain constant or accidental constituents may be responsible for symptoms not attributable to the original disease or to the toxicity of the drug. If an injury of the normal flora *per se*, or a secondary infection due to the loss of antagonistic activity can be harmful for the patient, the thought of a therapeutic substitution of the lacking normal flora may naturally arise.

In view of the present state of the problem, the relevant pathogenetical and therapeutic studies dictated by necessity evidently advanced more rapidly than our basic knowledge of the intestinal flora. Thus we are in agreement with the opinion of DEHNERT [1] who stressed that "The composition and capacity of the normal intestinal flora are as yet unknown... we have no suitable media for the cultivation of most species... endeavours for normaliz-

ing the damaged intestinal flora remain hypotheses until we are unable to define the normal flora".

Considering the several, often contradictory data, in order to supplement our previous investigations into the effect of antibiotics on aerobic enteric bacteria [2] and gain information on the human applicability of our relevant animal experiments [3, 4], it seemed desirable to perform a detailed analysis of the normal intestinal flora.

Material and methods

Faecal samples. Patients receiving no antibiotic treatment and free from enteric symptoms were selected. Samples were taken with cotton wool swabs from the faeces excreted in the morning. The time elapsing between sampling and cultivation was generally 3 (at most 5) hours.

Cultivation methods. Some centigram amounts of faeces were streaked onto a filter paper strip and weighed by means of a torsion balance. With a glass rod a suspension containing 10 mg per ml of faeces was prepared in saline. Then 0.5 ml aliquots of tenfold serial dilutions of the suspension were pipetted onto culture medium plates. Using sufficiently dried plates the above volume of suspension could be spread evenly without a glass rod. Bacterial counts were given as number of cells per mg faeces.

Anaerobic organisms were cultivated and counted on BIERKOWSKI's medium [5]. Anaerobic conditions were provided by the alkaline pyrogallol method of BUCHNER and PREISZ as modified by LERCHE and REUTER [6]. The reagent was placed in a paper container which was then fixed with adhesive tape to the lid of the petri dish. Thus the whole surface of the culture medium was utilized. Low O_2 tension was checked by cultivating *Cl. tetani* on control plates.

Anaerobic streptococci were grown on blood agar by use of the above method. Other more delicate organisms were cultured on blood agar incubated aerobically. Endo agar served for the cultivation of Enterobacteriaceae and for the counting of *Str. faecalis*. Counting of staphylococci, aerobic spore-bearing bacilli and saccharomycetes was performed on agar containing 7 per cent NaCl. Differentiation of Enterobacteriaceae strains was carried out on NÓGRÁDI's medium [7].

Other cultures were identified on the basis of morphological and staining properties.

Antibiotic resistance was examined by use of paper discs prepared in this institute by VINCENT's [8] method. The discs contained 30 μg penicillin, streptomycin, chloramphenicol, chlortetracycline or oxytetracycline, 22 μg sulphamethylthiazole or 100 μg neomycin. Erythromycin sensitivity was determined by the tube dilution method since the disc method gave no reliable results.

Statistical analysis. The value of χ^2 was estimated by the usual method. In case of values smaller than 5, the YATES correction was used. The level of significance was 5 per cent; lower levels were treated separately.

When it was possible, instead of the rough χ^2 test, STUDENT's "t" test was employed. Standard error was calculated by use of the equation

$$s = \sqrt{\frac{(x_i - \bar{x})^2}{n - 1}}$$

For comparing the standard error of bacterial counts, the F test was used.

In examining the frequency distribution of counts the standard error was calculated from the average of log values.

The data were arbitrarily grouped on the basis of relative percentage frequently obtained from intervals expressed in log units (Δx). Poisson distribution and goodness of fit of the observed to the theoretical frequencies were tested by *Drs.* T. LAKATOS and G. MASSZI.

Results

(1) *Examination of the normal intestinal flora.*

The faeces of 43 patients hospitalized with pyrexia were examined on 2 to 4 occasions. The patients received no antibiotics before and during the experiments and were free from enteric symptoms. Altogether 117 samples were examined. The distribution of the isolated microorganisms is presented in Table I. The averages (log $\bar{x}$) and physiological limits of bacterial counts were estimated from data obtained for individual patients and for repeated examinations, respectively.

From Table I it is seen that 4 different microorganisms, *E. coli*, *Str. faecalis*, Bacteroides and *L. bifidus* occurred in all patients and in all samples. The respective average counts for these bacteria were $10^{5\cdot4}$, $10^{4\cdot4}$, $10^{4\cdot9}$ and $10^{5\cdot1}$. *E. coli* and *Str. faecalis* were present in particularly varying numbers. Other bacteria as clostridia (mainly *Cl. perfringens)*, *Kl. pneumoniae* and the tribe Proteae were encountered in numbers amounting to $10^{4\cdot38}$—$10^{4\cdot75}$ cells/mg in 25—36 per cent of the samples. As to the order of frequency, staphylococci preceded Proteae (31 vs 25 per cent); the former bacteria, however, occurred only in small numbers ($10^{2\cdot2}$). Of fungi only yeast-like organisms were examined. These were found in low counts in 10 per cent of the samples. Aerobic spore-bearers occurred in numbers similar to those of staphylococci in 9 per cent

Table I

Constituents of the normal adult intestinal flora

Organism	Occurrence in				Average count per mg	Limits of count per mg
	43 patients		117 samples			
	No.	%	No.	%		
E. coli	43	100	117	100	$10^{5\cdot42}$	$10^{3\cdot0-6\cdot7}$
Kl. pneumoniae ..	18	42	40	34	$10^{4\cdot57}$	$10^{1\cdot0-6\cdot0}$
Proteae	16	37	29	25	$10^{4\cdot75}$	$10^{2\cdot4-6\cdot0}$
Pseudomonas	2	5	3	3	$10^{4\cdot40}$	$10^{3\cdot2-4\cdot6}$
Str. faecalis	43	100	117	100	$10^{4\cdot42}$	$10^{3\cdot0-7\cdot0}$
Staph. aureus.....	20	47	36	31	$10^{2\cdot23}$	$10^{1\cdot0-4\cdot2}$
Bacillus	10	23	11	9	$10^{2\cdot33}$	$10^{1\cdot0-5\cdot0}$
Clostridium	22	51	42	36	$10^{4\cdot38}$	$10^{3\cdot0-6\cdot0}$
Bacteroides	43	100	117	100	$10^{4\cdot98}$	$10^{3\cdot0-6\cdot0}$
L. bifidus	43	100	117	100	$10^{5\cdot11}$	$10^{3\cdot0-6\cdot4}$
L. acidophilus	12	28	21	18	$10^{4\cdot58}$	$10^{1\cdot0-5\cdot3}$
Saccharomyces....	6	14	11	10	$10^{2\cdot90}$	$10^{1\cdot0-5\cdot0}$

of the samples. Microaerophilic lactobacilli were relatively unfrequent (18 per cent). *Ps. aeruginosa* was isolated only from 2 samples originating from 2 patients.

The proportion of the occurrence of the four constant constituents and the commoner accidental bacteria is presented in Fig. 1.

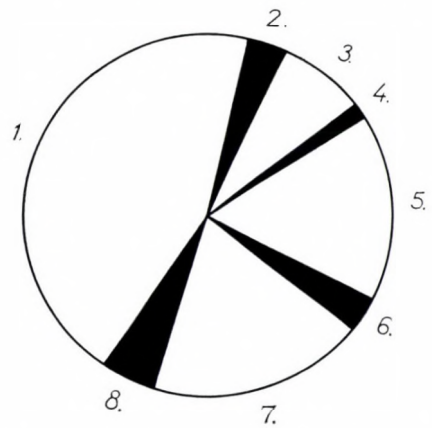

Fig. 1. Constitution of the normal adult intestinal flora 1. *E. coli* (46%); 2. *Klebsiella pneumoniae* (3%); 3. *Str. faecalis* (9%); 4. other (1%); 5. Bacteroides (16%); 6. Clostridia (3%); 7. *L. bifidus* (19%); 8. Proteae (4%)

In Fig. 1 white sectors indicate the constant, black sectors the accidental constituents. When calculating average values, the number of both patients and samples was considered.

The data show that the predominant organism of the faecal flora was *E. coli*, which occurred in higher counts than members of the anaerobic flora altogether. This surprising finding cannot be attributed to errors due to the cultivation technique. The absolute counts found for anaerobes (10^5/mg) were in agreement with data known from the literature (10^8/g). The explanation of this finding will be discussed later.

The individual variation in *E. coli* and *Str. faecalis* counts is presented in Fig. 2, with the relative frequency of counts expressed at 0.7 and 0.6 log unit (Δx) intervals, respectively. The standard errors were identical for both organisms ($S = 0.85$); the averages showed difference of one exponent. Owing to the high standard error, the histogram significantly differed from the theoretically calculated Poisson frequency distribution graph. The deviation was especially considerable at higher counts. An analysis of the type of distribution would obviously require more extensive examinations.

In Fig 3 the histograms for Bacteroides and *L. bifidus* are presented.

The average values and standard errors for these two constituents were similar. The standard error was considerably smaller than in the case of aerobic

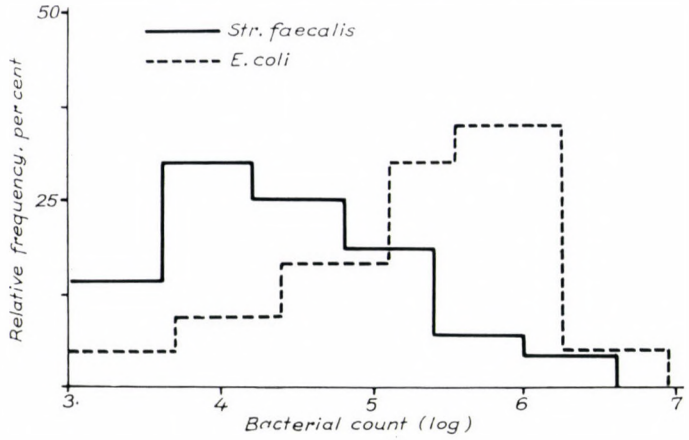

Fig. 2. Individual distribution of *E. coli* and *Str. faecalis* counts per mg faeces ($n = 43$) *E. coli*: $\bar{x} = 5.42$; $s = 0.85$; $\Delta x = 0.7$ log E, *Str. faecalis*: $\bar{x} = 4.42$; $s = 0.85$; $\Delta x = 0.6$ log E

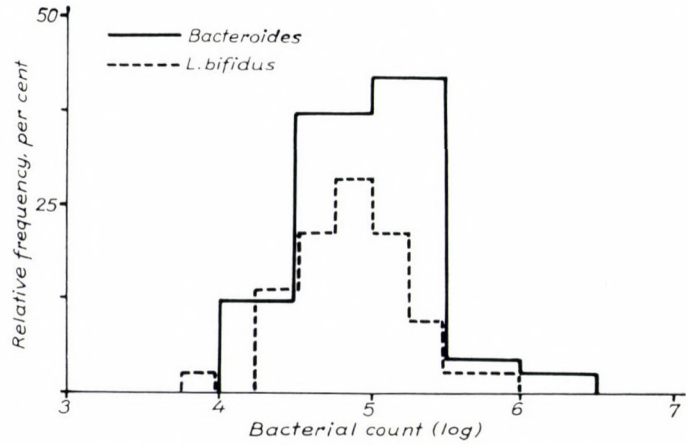

Fig. 3. Individual distribution of Bacteroides and *L. bifidus* counts per mg faeces ($n = 43$) *L. bifidus*: $\bar{x} = 5.11$; $s = 0.69$; $\Delta x = 0.25$ log E. Bacteroides: $\bar{x} = 4.89$; $s = 0.42$; $\Delta x = 0.5$ log E

bacteria. Thus the relative frequency was close to the average. The frequency distribution, mainly for Bacteroides, approached the Poisson graph, although testing the goodness of fit revealed some inhomogeneity for this group. Therefore, the determination of the frequency distributions of these organisms also requires more extensive studies.

The difference between the standard errors for aerobic and for anaerobic permanent constituents was notable:

$$F = \frac{s^2\,(E.\ coli + Str.\ faecalis)}{s^2\,(L.\ bifidus + Bacteroides)} = 2.214_{(42-42)}$$

which, at 1 per cent level, was significant statistically.

The high physiological or individual variation of viable counts indicates a considerable individual heterogeneity per se. In view of the small number of data it is not justified to establish various "types" of the intestinal flora. Nevertheless, in Table II such "types" are presented in order to demonstrate the high individual variation of bacterial counts. Thus it is clear that the "normal distribution" of members of the flora should be considered with appropriate caution.

Table II

Different "types" of the normal intestinal flora
Total number of cases, 39

Types	Average bacterial counts per mg				Total counts per mg	No. of cases
	E. coli	*Str. faecalis*	*L. bifidus*	*Bacteroides*		
1. "Balanced"	235 000 (45%)	21 000 (4%)	154 000 (30%)	108 000 (21%)	515 000	10
2. "Coli predominance"	1 566 000 (82%)	51 000 (10%)	182 000 (10%)	93 000 (5%)	1 882 000	14
3. "Streptococcus predominance"	169 000 (19%)	946 000 (61%)	77 000 (9%)	99 000 (11%)	1 289 000	5
4. "Lactobacillus predominance"	17 000 (6%)	10 000 (4%)	198 000 (73%)	48 000 (17%)	273 000	6
5. "Bacteroides predominance"	24 000 (5%)	6 000 (1%)	110 000 (23%)	330 000 (71%)	478 000	4

Among the 39 patients only 10 had an enteric flora which might be regarded as "balanced". In these patients the proportion of aerobic and anaerobic members was roughly similar. In 14 cases (82 per cent), *E. coli* predominated, but the absolute number of *L. bifidus* or Bacteroides was similar to that found in the first group. Thus the higher incidence of *E. coli* was due to an increase in the absolute number of this organism. The same holds true for the 5 cases in which *Str. faecalis* occurred as the predominating bacterium (61 per cent). In 6 cases without an alteration in the absolute number of bacteria, with very low *E. coli* counts, *L. bifidus* appeared in a high proportion (73 per cent). Finally in 4 cases with low *E. coli* and unchanged *L. bifidus* counts, Bacteroides occurred in definitely increased number and accordingly in higher proportions (71 per cent).

The association between the distribution of various bacteria and the age of patients is also interesting. As our materials were obtained mainly from older persons, only the results for 10 younger (aged under 50, average 25 years) and 10 older (aged 66 years on the average) patients were compared (Table III).

The viable counts shown in Table III represent average values for 10 persons of each group for both constant and accidental constituents.

It was striking that the only difference between the two age groups was that *E. coli* occurred in older persons in considerably larger numbers. The high *E. coli* count was responsible for the increase from 30 to 74 per cent in the proportion of these bacteria and for a three-fold increase in the total count.

Table III

Age group distribution of the normal adult intestinal flora

Organism	Age group			
	Under 50 (average 25) years		Over 60 (average 66) years	
	Cells/mg	%	Cells/mg	%
E. coli	100 000	30	700 000	74
Kl. pneumoniae	10	.	60	.
Proteae	60	0.02	300	0.03
Streptococcus	80 000	24	60 000	6
L. *bifidus*	70 000	21	90 000	9.5
Bacteroides	80 000	24	80 000	10
Clostridium	80	0.02	80	.
Total counts	330 142	100	950 440	100

(2) *Stability of the constant members of the normal intestinal flora.* Changes and restitution to the original of the flora of patients treated with antibiotics are to be discussed in a subsequent paper [9]. These experiments allowed to make some observations on the stability of constant constituents.

In these investigations the intestinal flora of 15 patients was examined before and after antibiotic treatment. The average values of the 4 constant constituents were compared and analysed by the "t" test. The striking result of these experiments was that, in contrast to the high individual variation and standard error mentioned above, the "t" test showed a considerable homogeneity within the same patient. The p values for the permanent constituents were, *E. coli* ~ 0.50; *Str. faecalis*, ~ 0.50; *L. bifidus*, > 0.70; Bacteroides, ~ 0.90.

Thus, although the number of these bacteria highly varied with different persons, in the same a considerable stability can be demonstrated. This stability is particularly conspicuous if the restitution of the disturbed equilibrium of the intestinal flora is taken into account.

The time needed for restitution is an important problem. Although it may depend on several other factors, for example on the antibiotic administered, Table IV offers some data showing the rapid restitution of the different constituents.

Table IV

Restitution of the normal flora during and after antibiotic therapy

Normal constituents*	Restitution		Chloramphenicol	Chlortetracycline	Erythromycin
E. coli	During treatment	Days	3	1.8	6.5
		Cases	7	5	2
	After treatment	Days	—	—	3.75
		Cases	—	—	8
L. bifidus	During treatment	Days	2.5	4.3	—
		Cases	4	3	—
	After treatment	Days	1.7	5.0	5.4
		Cases	3	4	10
Bacteroides	During treatment	Days	2.8	4.3	—
		Cases	4	3	—
	After treatment	Days	2.1	4.0	5.8
		Cases	3	4	10

* Because of the small number of cases *Str. faecalis* has been omitted

Str. faecalis, which is only exceptionally sensitive to antibiotics, has been omitted from Table IV. As to the remaining three constant organisms it is evident that complete stabilization occurred within 1 week. The equilibrium of *E. coli* was often restored during treatment, except when erythromycin was administered. In other persons the restitution of the *E. coli* flora took 4 days after antibiotic treatment had been discontinued. Restitution of the almost uniformly reacting anaerobic constituents was relatively slower. These organisms regenerated at an about equal rate during and after chloramphenicol or chlortetracycline treatment. When erythromycin was administered, restitution took 6 days after the therapy had been discontinued.

It was questionable whether the original predominant strains regained their predominance after treatment or were replaced by some antibiotic resistant cultures. Some estimations were made by examining the antibiotic sensitivity patterns of the isolated *E. coli* strains. Table V presents cases in which, on the basis of antibiotic patterns, it was possible to determine whether it was the original strain that persisted or a new strain had taken its place. In these experiments an observation described previously [2], has also been considered.

Out of the 26 cases included in Table V only 10 yielded new, mostly resistant strains during treatment. It is noteworthy that although in 7 patients new strains also appeared, later, in the post-therapeutic period these gave place to the sensitive form or gradually resistant mutants of the original strain.

Table V

Restitution of E. coli flora as estimated from the antibiotic sensitivity patterns of the isolated strains

Mode of restitution	Other *E. coli* strain appearing during treatment	Strain isolated after treatment	Antibiotic therapy					Total
			Su	St	C	T	E	
New strain	—	Sensitive	—	—	—	—	1	1
	—	Resistant	1	4	—	2	2	9
Restitution of the original strain	—	Sensitive	—	—	—	—	1	1
	+ (resistant)		—	—	1	—	3	4
	—	Resistant	1	2	2	—	3	8
	+ (resistant)		—	3	—	—	—	3
Total			2	9	3	2	10	26

Abbreviations: Su = sulphonamide, St = streptomycin, C = chloramphenicol, T = tetracyclines, E = erythromycin

Thus in the stabilization process 3 main possibilities can be considered.

(1) The old strain is replaced by a resistant new culture.

(2) Resistant mutants of the original strains become predominant.

(3) The transient predominance of a new resistant strain occurs during treatment. In the post-therapeutic period the original strain regains its predominance.

Discussion

As regards the constant members of the normal enteric flora, our data are in agreement with those in the literature. In addition to *E. coli*, *Str. faecalis*, *L. bifidus* and the heterogeneous Bacteroides group, some authors also include clostridia among the permanent members. MEISEL *et al.* [10] found *Cl. perfringens* in 93 per cent of a large number of samples. In contrast, in agreement with our findings, HAENEL [11] isolated these bacteria in approximately 50 per cent. There is no doubt that the discrepancies are due to differences in the technique used. The BIERKOWSKI medium employed in the present study is not selective, and is therefore only exceptionally suitable for the demonstration of organisms present in counts lower than 10^3/mg. Thus by the use of methods suitable for the detection of smaller numbers of clostridia, a higher incidence may be revealed.

According to HAENEL [12] members of the tribe Proteae are normal constituents of the intestinal flora, and these bacteria, although in very low counts, could be isolated from all members of a small group of patients. Our experience that Proteus tends to occur more frequently in certain periods, indicates that observation on the microecological position of the tribe Proteae should be evaluated cautiously and also that the excretion of Proteus

depends on alimentary factors. Our animal experiments supported this consideration [4].

Aerobic, or more precisely, microaerophilic lactobacilli are not regarded as normal members of the human intestinal flora [13]. According to LERCHE and REUTER [14] the occurrence of faecal lactobacilli is always associated with the *L. acidophilus* content of the mouth or food; thus these bacteria are of a secondary importance in the intestinal tract.

When selective media are used, staphylococci can be isolated with great frequency. In agreement with our findings, staphylococci were isolated by PRISSICK [15] in 45.8, by TODD [16] in 53, and by ANGYAL [17] in 44 per cent. Similarly to our results, HAENEL [11] gave the average staphylococcal count as 10^4/g faeces.

As to the number of anaerobic constant constituents, our findings agree with those known from the literature. For *Str. faecalis* and especially for *E. coli*, higher values were obtained. This discrepancy appeared in a shift in the distribution of the normal constituents. According to HAENEL [18] *L. bifidus* constitutes 45 to 70 per cent of the flora. Other authors described the Bacteroides group as a predominant representative of human intestinal flora [19]. The results agree in that the aerobic members constitute only 1 to 20 per cent of the total counts. In surveying our observations an explanation of this discrepancy between our and the other authors' data was revealed. The relatively high *E. coli* and *Str. faecalis* counts, the higher standard error and deviation in the frequency distribution at higher counts may be explained by the difference in the age of the examined patients. Most of our cases (almost 70 per cent) were of the older age group. These patients did not suffer from enteric symptoms but spent most of their time in bed, and thus were more or less likely to have passage disorders. HAENEL [20] mentioned an *in vitro* type of dysbiosis appearing in faecal samples kept at room temperature or sent to the laboratory by mail. In such samples the number of anaerobic bacteria decreased, while that of *E. coli* and some cocci increased. In our opinion prolonged intestinal passage exerts a similar effect. The multiplication of sensitive anaerobes decreases or ceases although due to low redox conditions the organisms remain viable. In contrast, *E. coli* and *Str. faecalis* multiply freely and cause a shift in the distribution of the constituents. As the passage disorder presumably influences only the number of aerobic organisms, it has been concluded that our data do not reflect the normal human intestinal flora, but merely the normal flora of patients staying in bed. At the same time, our studies yielded valuable data concerning the so-called dysbiotic flora resulting from antibiotic therapy. Our findings confirm the opinion that eubiosis or dysbiosis should be interpreted with great caution.

As regards constant constituents, a notable stability of counts has been revealed even in cases of a highly disturbed equilibrium. Restitution

of the original flora took place within the remarkably short period of at most one week.

The stability of bacterial counts and the rapid capacity to restitution call the attention to two important factors. The mechanism of the phenomenon, which cannot be explained by a simple antagonism among normal constituents, is an important question. If the factor responsible for the restitution of the original strain, as in our examples for that of *E. coli*, is also considered, our earlier hypothesis [3, 4] of there being close association between *E. coli* and perhaps also the other constant constituents and the intestinal mucosa of the host has definitely been supported.

The other factor is of practical interest and concerns "substitution" therapy, in other words the artificial replacement of the damaged normal flora. In view of the restitution capacity and stability of the original strains, the success of such treatment seems doubtfull. In agreement with the authors criticizing the effectiveness of this therapy [21, 22, 23, 24], on the basis of the present results the "substitution" nature of "substitution therapy" should be denied.

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STUDIES ON THE NUCLEIC ACID METABOLISM OF CHORIOALLANTOIC MEMBRANE CELLS AFTER INFLUENZA VIRUS INFECTION

By

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Summary. The virological and biochemical events taking place during the first cycle of virus reproduction in the chorioallantoic membrane of 12-day-old chick embryos infected with the Singapore 1/57 strain of influenza A-2 virus have been studied. The points of time when soluble antigen, haemagglutinin and infectious virus appeared, have been established. The velocities of nuclear RNA and protein synthesis markedly increased after infection. The mechanism of the effects on the cell of different RNA viruses is discussed.

It is well-known from recent reports that in the presence of synthetic polynucleotides polypeptides of defined amino acid sequence are produced in ribosomal systems, *in vitro* [1, 2, 3]. In *Escherichia coli* ribosomal systems the synthesis of virus-specific polypeptides has been observed by TSUGITA *et al.* [4] and OFENGAND *et al.*[5], using tobacco mosaic and turnip yellow mosaic virus ribonucleic acid (RNA), respectively.

As to the possible mechanism and intracellular localisation of the virus or viral RNA replication there is a certain discrepancy of opinion. Some authors suppose the replication to take place exclusively in the cytoplasm [6, 7], while others describe the phenomenon as a primarily intranuclear event in which the cytoplasm is involved only secondarily [8, 9, 10, 11]. Specific inhibitors of the synthesis (or the functions) of different kinds of intracellular RNAs may successfully be used in studies on the mechanism of viral RNA replication. For example, Actinomycin D is an effective inhibitor of DNA—RNA transcription, *i.e.* of messenger RNA synthesis [6]. This substance has no effect whatever on the replication of polio and EMC viruses [6, 12, 13, 14] and thus suggests these processes to be independent of the nuclear RNA synthesizing mechanisms of the cell. On the other hand, the soluble antigen of influenza virus accumulates in the nucleus in the early phase of infection, as it has been revealed by electronmicroscopic studies [15].

Therefore it appeared interesting to investigate in one-step growth experiments the replication of influenza A-2 virus (strain Singapore 1/57) by examining the time sequences and quantitative relations in the appearance of soluble antigen, haemagglutinating factor and infective virus particles. The results were evaluated on the basis of simultaneous examination of the metabolism of certain biopolymers in the host cell and in its various subcellular fractions.

Materials and methods

One-step growth experiments. Embryonated eggs incubated for 12 days were inoculated with 0.3 ml of Singapore 1/57 strain of influenza A-2 virus in a suspension containing $10^{7.5-8}ID_{50}$ per ml, purified by centrifugation at 20 000 *g*. This relatively large inoculum was used in order to achieve a simultaneous infection of nearly all susceptible cells of the chorioallantoic membrane. At various intervals after infection the membranes were removed, washed three times in saline, and treated with 80 haemagglutination-inhibiting (HI) units of immune serum to eliminate residual, adsorbed virus particles. After washing three more times with saline solution to eliminate the immune serum, the membranes were homogenized in a Waring-blendor in 2 volumes of saline. After centrifugation at 1200 *g* for 5 minutes the supernatant was carefully removed and used for virological tests.

Soluble antigen was measured by the complement fixation (CF) reaction with anti-s human serum. The highest dilution of antigen giving a positive reaction with 4 units of specific antibody was taken as end point. Both the complement fixation and the haemagglutinin titrations were performed by standard methods in a Microtitrator apparatus [16]. Infectivity titre was measured by the roller-drum method described by HORVÁTH [17].

Biochemical methods. The nucleic acids were separated by a previously described modification [22, 23] of the SCHMIDT—THANNHAUSER [18, 19] method. The RNA and DNA contents were measured by the orcinol [20] and indole [21] colour-reactions, respectively.

Prior to infection, the nucleic acids of the host cells were labeled for 1 hour at 37° C by 40 μC ^{32}P/egg in the form of carrier-free $KH_2{}^{32}PO_4$. At various intervals after infection, the chorioallantoic membranes from groups of 6 eggs each were removed, washed with saline at 4° C and stored at —70° C.

The membranes were then thawed and homogenized in distilled water at 4° C. The homogenate was treated with 0.5 volume of 30 per cent TCA solution. After centrifugation at 700 *g* for 15 minutes in a ZG type refrigerator centrifuge, the sediment was washed six times with 10 per cent TCA. There was no detectable radioactivity in the 6th supernatant. The precipitate was extracted successively with ethanol, ethanol-chloroform 3 : 1, ethanol-ethylether 3 : 1, at 50° C, and dried with ether. The nucleic acids were extracted by the method of DAVIDSON and SMELLIE [24] from the dry powder and were incubated in *N* NaOH at 37° for 18 hours. The solution was then acidified with 30 per cent TCA and centrifuged to separate the RNA-nucleotides (supernatant) and DNA (precipitate). The DNA fraction was treated once more with *N* NaOH (1 hr. at 40° C) and reprecipitated by TCA. The nucleic acid content and the radioactivity of each of these fractions were then determined. Radioactivity was expressed as cpm/100 μg nucleic acid phosphorus (specific activity).

In other experiments nucleic acids were prepared by treatment with 70 per cent phenol according to KIRBY [25] as described previously [22, 26]. For these studies membrane homogenates from groups of 15 eggs labeled with ^{32}P as described above, were used. The specific activity of "phenol-released" RNA was measured after purification on an Ecteola-Cellulose column (0.41 meq/g, SERVA). After adsorption, the column was washed with 0.01 *M* phosphate buffer (pH 6.85) containing 0.2 *M* NaCl until no further activity could be eluted. The RNA was then eluted with 0.01 *M* phosphate solution containing 1 *N* NH_4OH. The phenolic phase containing the "residual" RNA fraction was treated with 4 volumes of ethanol [27] in the presence of 2 per cent potassium acetate. Extraction and determination of nucleic acids in the resulting precipitate were carried out as described above.

For the labeling of proteins, ^{14}C-lysine (Radiochemical Centre, Amersham, England) was applied in a dose of 2 μC/egg. The chorioallantoic membrane homogenates labeled for various periods after infection were separated into nuclear and cytoplasmic fractions with glycerol, according to the method described previously for Lettré-Ehrlich ascites tumour cells [23]. The fractions obtained were controlled by phase-contrast-microscopy, and by calculation of their RNA/DNA ratios. The nuclear fraction was extracted with *N* HCl. The ^{14}C-activities and protein contents were measured in the acid-soluble and acid-insoluble fractions derived from the nuclei, as well as in the homogenate and the cytoplasmic fraction. Protein was estimated by LOWRY's method [39], the activities were expressed as cpm/mg protein.

Radioactivity of ^{32}P was measured by Orion EMG 1872 decimal scaler using a 1.5 mg/cm^2 end window GM tube. ^{14}C-activity was measured by a Friesecke-Hoepfner 2 methan flow counter. The colorimetric and spectrophotometric measurements were performed in the Unicam SP 500 spectrophotometer.

Results

The intracellular cycle of influenza virus replication was studied in one-step growth experiments by determining the rates of production of CF antigen, haemagglutinating and infective particles. The average values of parallel determinations were plotted as a function of time after the infection (Fig. 1).

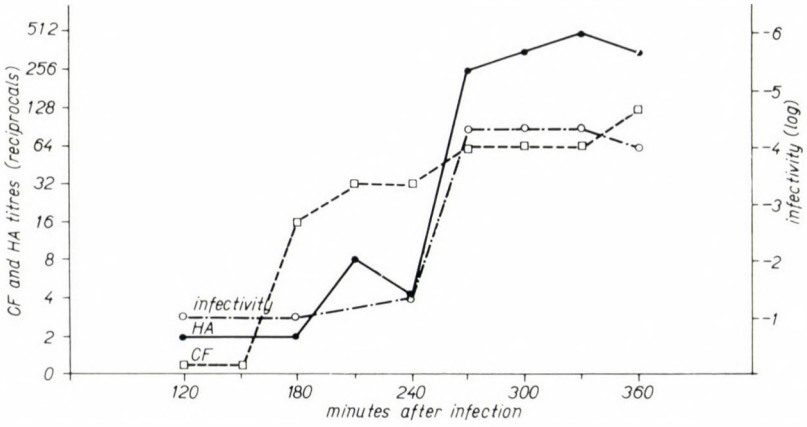

Fig. 1. Infective, HA and CF titres obtained at various times after infection with influenza A-2 virus

In chorioallantoic membrane homogenates no soluble antigen could be detected in the first 2.5 hours following infection. In the 3rd hour, however, a sharp increase was observed, which continued at a decreasing rate up to the 6th hour. The haemagglutinin and the infective titres remained unchanged at the low initial level of the first 4 hours. After 4.5 hours a simultaneous increase of the haemagglutinin and infective titre was observed, this persisted throughout the additional 1.5 hours of observation.

The CF activity of influenza virus is characteristics of the soluble antigen known to be of ribonucleo-protein nature. As this material was the first viral component demonstrable in infected cells, it seemed to be of interest to compare the RNA content of infected and uninfected cells at various times after infection. No significant quantitative difference could, however, be found using the SCHMIDT—THANNHAUSER method. Both the infected and the control cell samples contained 515 μg of RNA and 231 μg of DNA per 100 mg of dry substance. The ratio of RNA/DNA was 2.26 ± 0.25 for both.

The rate of nucleic acid synthesis in infected and control cells was studied by ^{32}P incorporation (Fig. 2).

At the 45th minute following infection, the specific activity of total RNA was considerably lower in the infected than in the control cells. From

45 min. to 270 min. there was a 4—5fold increase of ^{32}P incorporation in the infected cells as compared to the controls. The specific activity of DNA in the infected cells reached a level 4—5 times higher than that of the controls after about 180 minutes and this difference persisted for the rest of the experiment.

An attempt was made to specify more precisely the RNA fraction responsible for the increased incorporation of ^{32}P in the infected cells. The

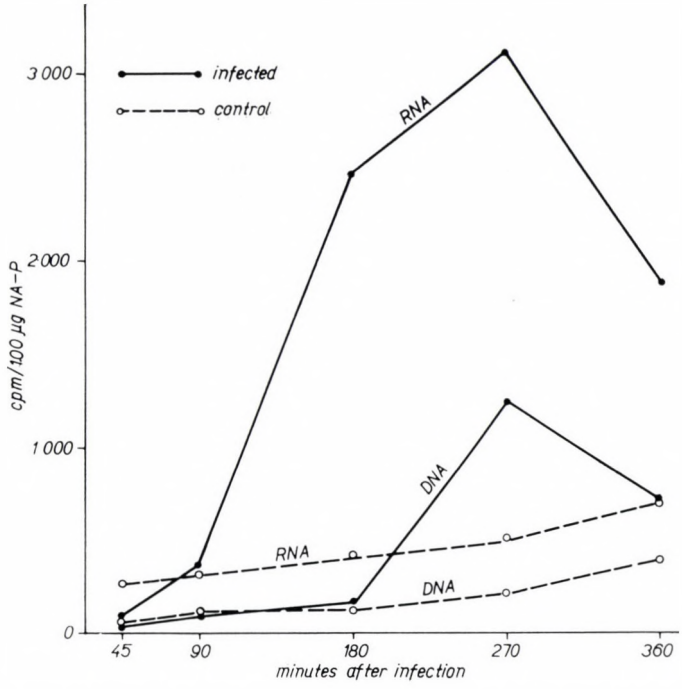

Fig. 2. Specific activity of RNA and DNA preparations derived from infected and non-infected chorioallantoic membranes

membrane homogenate was treated with 70 per cent phenol and the specific activities of "phenol-released RNA" and of "residual RNA" were determined at various points of time following infection (Fig. 3).

Forty-five minutes after infection the specific activity of "residual RNA" was lower in the infected than in the control cells. From 45 minutes the specific activity of this fraction from infected cells exhibited a marked increase and reached its maximum at 90 minutes, when it was ten times that from the control cells; beyond 90 mins. it showed a slight decline. In contrast, the specific activity of "phenol-released RNA" did not display any increase until 180 minutes after infection and the maximum value was considerably lower than that of the "residual RNA" fraction.

The "residual RNA" fraction is known to be mostly of nuclear origin, and as it was the first to exhibit an increased specific activity after virus infection, the specific activities of RNA of isolated nuclei were determined at different intervals. The RNA/DNA ratio in the nuclear fractions obtained

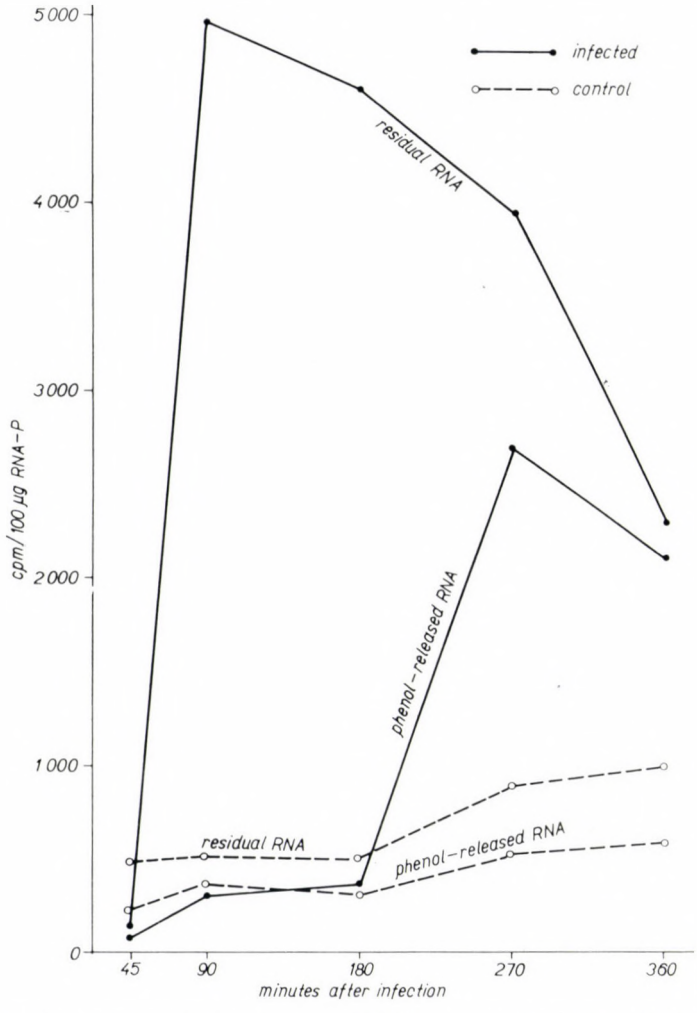

Fig. 3. Specific activity of phenol-released-RNA and residual-RNA derived from infected and non-infected chorioallantoic membranes

by the glycerol method was 0.62 in the infected as well as in the control cells. The specific activities of the nuclear and "residual" RNA fractions went parallel during the first 6 hours of the cycle; the former was, however, always higher than the latter.

On the basis of these results, and since it is known that nuclear proteins (histones and protamines) are rich in basic amino acids, the incorporation of ^{14}C-lysine into the proteins of various subcellular fractions was studied after infection (Table I).

Table I

^{14}C activity in various fractions of infected and control chorioallantoic membranes

Minutes after infection	CAM	Homogenate	Cytoplasm	Nuclear	
				HCl-soluble	HCl-insoluble
45	control	27*	28	20	32
	infected	31	26	41	61
90	control	65	41	124	123
	infected	1119	1210	976	1137
180	control	715	628	588	930
	infected	1515	1925	1685	2442

* cpm/1 mg protein = ^{14}C lysine.

Table I shows that lysine incorporation into the nuclear proteins (acid-soluble and -insoluble fractions) of infected cells exceeded that of the controls at the 45th minute of the cycle. Incorporation into cytoplasmic proteins was similar in both the infected and the control cells. Later on, however, there were no detectable differences between the specific activities of the cytoplasmic and nuclear proteins, although the values in the infected samples were always higher than in the controls.

Discussion

In RNA-viruses the complete genetic information necessary for the synthesis of the virion is carried in the viral RNA. The localisation of synthetic processes involved in the replication of various types of RNA-virus is not clear [28]. Information available in the viral RNA might (a) be translated directly, in the manner of messenger RNA, by the ribosomal system of the cells [29, 30, 31]; (b) exert an action on the nuclear "normal" messenger synthesis of the host; or (c) initiate virus replication by some action on the genetic material of the host cells [32, 33].

In the present study the mechanism of replication of an RNA virus has been examined in one-step growth experiments. The appearance of certain biological characteristics and biochemical events was studied during the first cycle of influenza virus replication.

We found that the CF antigen, i.e. the nucleocapsid, of influenza virus appeared in the 3rd hour following infection. A similar observation was made by BREITENFELD and SCHÄFER [34] with fowl plague virus. These authors succeeded in detecting the soluble antigen in the 3rd hour of infection in the nuclei of the host cells, by the fluorescent antibody technique.

The appearance of infectivity was approximately simultaneous with that of the haemagglutinin and both continued to accumulate during the next 1.5 hours. Similar relations in the order of appearance and in the localisation of viral components were found by HOYLE [35, 36, 37] and LIEF [38] for the PR8 strain of influenza.

The amount of soluble antigen showed a slow continuous accumulation after the first rapid increase. This is in contrast with the kinetics of virion production; thus one might suppose that viral RNA is produced for a longer period and in larger amounts than necessary for the total virus yield by the cell.

The kinetics of labeling (^{32}P) of the "residual RNA" fraction in infected cells and that of the RNA fraction in isolated nuclei suggested that nuclear synthesis of RNA preceded the appearance of the nucleocapsid. This observation is in agreement with an electronmicroscopic study of the production of influenza virus [15] and supports the view that the nucleocapsid is synthesized in the nucleus of the host cell.

In the 45th minute the radioactivity of RNA was lower in the infected than in the control cells. This observation suggests an inhibition of RNA synthesis by infection and a breakdown of "normal" RNA before the beginning of viral RNA replication.

In infected cells the labeling of the "phenol-released" RNA fraction started to increase in the 180th minute of the cycle. The appearance of haemagglutinin and infectious virus in the cytoplasm coincided with the increase of labeling of the cytoplasmic ("phenol-released") RNA. This fact in addition to those presented above favoured the supposition that the soluble antigen was formed in the nucleus, while the other components were synthesized in the cytoplasm.

As to the observed changes in DNA synthesis of infected cells we believe that these were not specifically related to the viral cycle, because (1) the phenomenon was a rather late one (after 3 hours); (2) the intensive labeling of nuclear RNA might have caused some contamination of the DNA fraction in spite of the double alkaline hydrolysis and precipitation applied; (3) the possible early inhibition of DNA synthesis in the infected cells might have terminated at a certain phase of the cycle, and this recuperation of DNA synthesis might have caused the increased activity observed.

The study ^{14}C-lysine incorporation into the proteins (Table I) produced by the infected cells has shown that in the 45th minute only the acid-soluble and acid-insoluble proteins of the nuclei were more highly labeled than the

controls, while in the 90th minute the activity was present in every fraction of the cells tested and was considerably higher in the infected than in the control samples. This increased level of protein synthesis may serve for the production of viral RNA polymerase and for that of other viral proteins.

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THE EFFECT OF DIFFERENT ANTICANCER AGENTS ON INDUCIBLE SYSTEMS OF BACILLUS MEGATERIUM

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Summary. The production of bacteriocin (megacin) by *Bacillus megaterium* is elicited by ultraviolet irradiation and reincubation of the culture of megacinogenic strain, *i.e.* by induction of bacteria. The elaboration of megacin therefore resembles lysogeny. The aim of the present study was to ascertain whether megacinogeny could be induced by chemicals since the lysogenic strains of *B. megaterium* is also inducible by certain compounds. Twenty different compounds were tested on a megacinogenic strain and, for comparison, on a lysogenic strain of *B. megaterium*. Some of the compounds tested were anticancer agents. Only chemicals capable of inducing lysogeny were found effective in inducing megacinogeny. No general rule could be established concerning chemical structure and effect. Even compounds closely similar in structure, such as the ethylenimines, behaved differently as regards induction. Some of them were ineffective while others induced both megacinogeny and lysogeny. This observation suggests that the receptors in cells are not identical even for similar alkylating agents.

The present study yielded additional support of the hypothesis that megacinogeny is governed by a highly defective prophage.

In studying strains of *Bacillus megaterium* isolated from soil samples, a particular phenomenon was observed in this laboratory some years ago. A proportion of strains cultivated in yeast-extract peptone broth exhibited unusual growth curves [1]. After an initial logarithmic period, the optical density of cultures decreased and the bacteria finally lysed. In some cases, the initial lysis was followed by a secondary growth and the culture reached the stationary phase. Although the phenomenon resembles lysogeny, no infective phage particles could be detected. Instead, an antibacterial principle had accumulated in the cultures. The antibacterial substance was not sedimentable in a preparatory ultracentrifuge; it was inactivated by proteolytic enzymes and did not dialyze through cellophane [2]. In view of its narrow antibacterial spectrum the protein character of the antibacterial substance, the principle was classed among bacteriocins and was named megacin [2]. Megacin of strain 216 was isolated by HOLLAND [3] in homogeneous form and appeared to be a simple protein of 51 000 molecular weight.

Spontaneous lysis of megacinogenic bacteria occurred only in certain media. A slight dose of ultraviolet irradiation is usually required for the lysis of organisms associated with megacin liberation. Although megacinogeny simulates lysogeny in several respects, not even incomplete phage structures were revealed in lysates of megacinogenic bacteria by electronmicroscopic

study [4, 5]. A hypothesis has been advanced [6] that megacinogeny may be due to a highly defective prophage which cannot be recognized since it does not yield the typical product of lysogeny. In fact, no direct evidence of this hypothesis could be secured so far. Attempts at isolating megacinogenic clones from typical lysogenic strains have failed [7].

As demonstrated by LWOFF *et al.* [8, 9, 10], a number of different chemicals are capable of eliciting induction in lysogenic bacteria. In their experiments carried out with a lysogenic *Bacillus megaterium* strain, the results of individual trials varied according to the culture medium. They mostly used a yeast extract medium, the standardization of which could hardly be achieved. We found that a well-defined synthetic medium could be supplemented for the complete medium in inducing both lysogeny and megacinogeny.

The aim of the present study was to ascertain whether or not the induction of megacinogeny could be effected by chemicals, and, if so, whether the different chemicals inducing lysogeny and megacinogeny exhibited a similar or a different pattern.

Materials and methods

Strains. Megacinogenic strain 216 was described earlier [2]. *B. megaterium*, strain 899 lysogenic with phage 1, and the phage, — sensitive strain Mutilate originated from *Prof.* A. LWOFF's laboratory. Strain Mut-C is a phage-resistant but megacin-sensitive derivative of strain Mutilate [11].

Media. Synthetic medium (SM): NH_4Cl, 2 g; K_2HPO_4, 1.4 g; KH_2PO_4, 0.6 g; Sodium citrate, 1 g; $MgSO_4$, $7H_2O$, 0.05 g; $MnSO_4$, 4 H_2O, 0.05 g; distilled water, 1000 ml; pH adjusted to 7.2. Before use, 2 ml sterile 1 per cent solution of Aminosol pulvis (Vitrum, Stockholm), an acid casein hydrolysate, and 2.5 ml of 20 per cent glucose was added to each 100 ml of medium.

YP (yeast extract peptone) medium was prepared as described earlier [2].

Induction of the bacteria. Earlier papers from this laboratory [2, 12] contain the technical details of the induction of megacinogenic bacteria.

10 ml SM was inoculated and incubated at room temperature overnight. 100 ml Erlenmeyer flasks containing 10 ml SM each were inoculated with 0.2 ml from the overnight culture and the flasks were incubated under gentle shaking in a water bath at 37° C. Induction of bacteria with chemicals or ultraviolet light was carried out when the optical density of the exponentially growing cultures had reached 0.2 (approx. 10^7/ml colony formers). Various concentrations of the chemicals tested were added to a set of cultures and these were then reincubated.

The bactericidal and bacteriostatic concentration of individual compounds varied considerably. Preliminary tests were, therefore, made to establish their bacteriostatic concentration. The inducing effect of the chemicals was investigated near to the range of their bacteriostatic concentration. Induction of megacinogeny or lysogeny was considered when in a culture partial or mass lysis ensued, associated with liberation of megacin and phage, respectively. Chemicals causing induction elicited bacterial lysis 1 to 3 hours after their addition to the cultures. Each culture was centrifuged after 6 hours reincubation and they were assayed for megacin or phage. Lysogenic strain 899 [1] liberated 10^5—10^5/ml phage particles without induction of bacteria in individual experiments. A 2 to 4 log unit increase in phage yield was accepted to be significant for the inducing effect of a chemical.

When megacinogenic strain 216 was grown in synthetic medium, no or only traces of megacin could be detected in the culture fluid. A titre of megacin of at least 1 : 1000 was taken as the criterion of induction. Usually 10^{-4} to 0.5×10^{-4} dilutions gave a clear halo on the indicator lawn.

Assay for phage particles were made on YP agar plates with phage-sensitive bacteria in the soft agar layer. Titration of megacin was carried out by dropping various dilutions of lysate onto the surface of YP agar plates seeded with Mut-C bacteria.

Results and conclusions

Twenty different chemicals, several of them with known antitumour effect are listed in the Table I.

Table I

The inducibility of megacinogenic (strain 216) and lysogenic (899) strains of Bacillus megaterium by different chemicals

Group of chemicals	Substance investigated	50% inhibition μg/m	Induction of strain		Effective[1]) concentration μg/ml
			216	899	
β-chlor-ethyl-amines	N-mustard	100	+	+	60—160
	Sarkolysin	1 000	—	—	
	Endoxan	>4 000	—	—	
Ethylimines	Bayer E 39	100	—	—	
	Trenimon	5	+	+	3—7
	TEM	800	+	+	400—600
	C 69 (Hoechst)	>1 000	—	—	
Antibiotics	Sarcomycin	10 000	—	—	
	Actinomycin-C	5	—	—	
	Mitomycin-C	0.5	+	+	0.3—1
	Carcinophilin	10 U[2])	+	+	2—12 U.
Miscellaneous	H_2O_2	20	+	+	5—30
	Ethyl-methanesulfonate	10 000	—	—	
	N-methyl-N′-nitro-N-nitroso-guanidine	50	+	+	10—15
	Hydroquinone	60	—	—	
	Benzoquinone	10	—	—	
	6-mercaptopurine	1 000	—	—	
	β-phenyl-ethyl-alcohol	3 000	—	—	
	Colcemid	30	—	—	
	Vinkaleukoblastin	100	—	—	

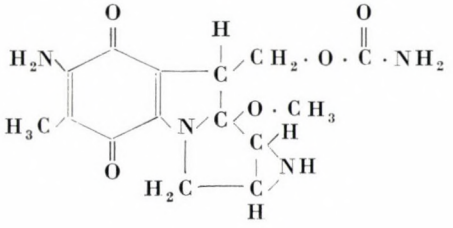
[1] Lowest and highest concentration causing marked induction. Substances which did not exhibit an inducing effect were tested within a broad range of concentrations; e.g. Sarkolysin : 2—1200 μg/ml; Bayer E 69 : 10—300 μg/ml; β-phenyl-ethyl-alcohol: 500—20 000 μg/ml

[2] U = unit

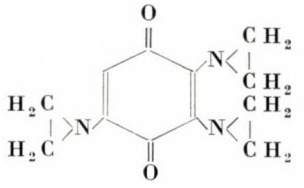
Some of the compounds were alkylating agents. Others, such as β-phenyl-ethyl-alcohol were inhibitors of DNA synthesis [13, 14]. The marked mutagenic effect of ethyl-methane-sulphonate has been exploited by several authors for genetic investigations of bacteria since LOVELESS' [15] systematic studies of the compound. The potent mutagenic action of N-methyl-N'-nitro-N-nitrosoguanidine [16] was found to be cancerostatic in the mouse [17].

While most of the anticancer drugs tested were alkylating agents, some of the substances like Vinkaleukoblastin [18], and Colcemid (desacetyl-methyl-

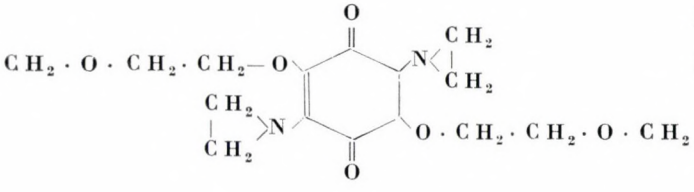
colchicin), were alkaloids. The molekula of Mitomycin-C also contains a reactive alkylating group (see Fig. 1). The chemical structure of the highly effective antibiotic Carcinophillin has not been yet established. A number of well-known compounds (benzoquinone, H_2O_2) were also tested for comparison.



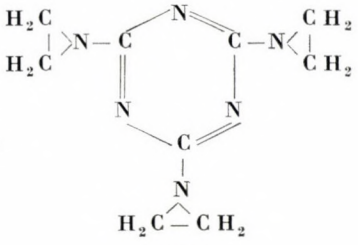
Mitomycin



Trenimon



Bayer E 39



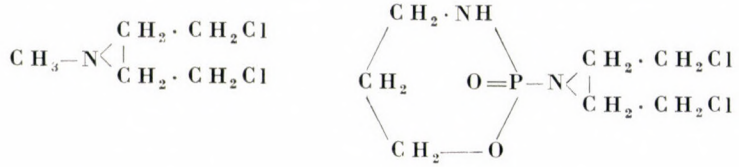
TEM

Fig. 1

The bacteriostatic concentration of the investigated compounds varied between 0.5 to 10 000 μg/ml. Accordingly, the effective concentration of compounds capable of inducing both lysogeny and megacinogeny lay within a broad range. All chemicals causing induction of lysogeny were also capable of inducing megacin production. None of the chemicals investigated showed an asymmetry in induction of the two systems. This strict parallelism suggests a similar genetic background for the two inducible systems of *Bacillus megaterium*. Beside our earlier observations e.g. the high segregation rate of non-megacinogenic clones [5] from megacinogenic strains, the present observations suggest that megacinogeny is governed by an episomic element, probably a highly defective prophage. The defectivity of the prophage does not allow the production of morphological structures of phage particles, and only some integrants of phage protein are produced.

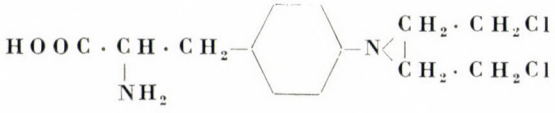
The nature of prophage induction process in lysogenic bacteria is still obscure although, in agreement with the known data, induction can be brought about by treatment which selectively inhibits DNA synthesis. Substances similar in chemical structure may apparently exert diverse effects when induc-

ing lysogeny. N-mustard was found capable of inducing both lysogeny and megacinogeny while some of its derivatives were devoid of this action. As to the cause of this difference, it could be supposed that Sarkomycin and Endoxan prefer to alkylate less vital receptors of cells than N-mustard does. Our findings indicated, further, that individual alkylating agents may act differently. This was in accordance with the observations concerning ethylenimines. It can be seen that closely related compounds (see Fig 1 and Fig 2) like Trenimon, Bayer E 39, and TEM, exhibited diverse effects in induction experiments.



N—mustard

Endoxan



Sarkolysin

Fig. 2

LEIN *et al.* [19] recommended a method for detecting potential antitumour agents by lysogenic bacteria. They used *Escherichia coli* K12, lysogenic with lambda, and tested a considerable number of antibiotics and fermentation products of microorganisms. Several antibiotics with known antitumour action such as mitomycin, azaserine, gancidin, grisealutein, streptonigrosine *etc.* were found effective in inducing lambda phage. 125 other antibiotics not specified in their paper gave negative results. In our observations with *B. megaterium* carrying inducible episomic elements no strict correlation was found between the chemical structure and the inducing effect of the investigated compounds. Yet, the simple technique of experiments with lysogenic bacteria deserves interest as one of the tools for screening antitumour agents.

Acknowledgements. We are indebted to *Prof.* B. KELLNER and *Dr.* L. NÉMETH for supplying some anticancer agents; and to Farbwerke Hoechst A. G., and Bayer A. G., Leverkusen, for some compounds used in these experiments.

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MYCOLOGICAL EXAMINATION OF CLINICAL MATERIALS

By

I. VITÉZ and E. K. NOVÁK

State Institute of Hygiene (Director: T. BAKÁCS), *Budapest*

(Received January 27, 1964)

Summary. From different clinical materials 63 fungal strains were isolated and identified. The examinations yielded further data for the fungal flora of patients. It has been pointed out that fungi are not always secondary invaders in pulmonary infections but may primarily be responsible for conditions resembling tuberculosis.

For a successful prevention and treatment of the more and more frequently occurring deep and surface mycoses, the introduction of mycological examinations in bacteriological laboratories would be desirable. Rapid and precise diagnosis is indispensable for prescribing an adequate therapy.

The present investigations were started in view of these considerations by one of the authors (I. V.) at the Institute of Hygiene, University Medical School, Budapest. In the course of the examinations 63 fungal strains were obtained, including cultures isolated first in Hungary from generalized lesions (nocardiosis [1] and candidiasis due to *Candida krusei* [2]). These strains were identified in the State Institute of Hygiene, Budapest, by the other author of the present paper (E. K. N.).

Materials and methods

The majority of the samples had been sent to the laboratory by clinicians. Some samples, *e.g.* those from onychomycosis, had been taken by the authors. The specimens were inoculated into fluid and onto solid media described by VITÉZ [3] and NOVÁK [4]. After incubation at 26°C morphological examination of the cultures was performed in preparations stained with toluidine blue. Pure strains were obtained by streaking highly diluted cultures onto solid media. The pure culture was examined for mouse pathogenicity. The growth of 48—72 hour agar slants was homogenized in 5 ml saline. Of the suspension 0.5 ml aliquots were injected intraperitoneally into each of 3 white mice weighing 16—18 g. After death of the animals, the corresponding strain was recovered from the heart blood. The mice were usually observed for 7—10 days.

Prior to identification, the cultures were checked for purity on the triphenyltetrazolium chloride agar described by PAGANO, LEVINE and TREJO [5]. Identification was carried out by the method of LODDER and KREGER-VAN RIJ [7] modified by ZSOLT and NOVÁK [6]. Determination of the taxonomic position of strains was based on the system of NOVÁK and ZSOLT [8].

Results and discussion

The distribution of materials from which the cultures were isolated was as follows. Sputum, 11; bronchoscopic sample, 9; laryngeal swab, 8; necropsy material, 18; nail-scraping (hand), 9; nail-scraping (foot), 2; throat swab, 1;

Table I

Fungal species isolated from clinical materials

Serial number	Species	Mouse pathogenicity	Sputum	Bronchoscopic sample	Laryngeal swab	Nail-scraping (hand)	Nail-scraping (foot)	Necropsy material	Throat swab	Pus	Culture	Urine	Total
1	*Azymocandida corniculata*....	—			1							1	1
2	*Candida sp., C. benhamii** ...	+				1		2					3
3	*Candida* sp., *C. beverwijkii** .	—					1						1
4	*Candida claussenii*.....	+	3	1	1			2					7
5	*Candida famata*	—				1							1
6	*Candida krusei*.....	—						3	1				4
7	*Candida norvegensis*	+				1							1
8	*Candida pseudotropicalis*	—		1	1								2
9	*Candida sp.*	+	1								1		2
10	*Candida solani*	+				1							1
11	*Cryptococcus albidus*	—				1							1
12	*Cryptococcus neoformans*.....	+	1										1
13	*Fung. imperf. sp.*	—				1							1
14	*Geotrichum candidum*	±	2			1							3
15	*Nocardia asteroides*	—						1		1			2
16	*Paratorulopsis bánhegyii*	—			1	1							2
17	*Paratorulopsis pinus*	—	1								1		2
18	*Penicillium sp.*	—		2	1								3
19	*Procandida albicans*	+		3	3			7			1		14
20	*Procandida majoricensis*	+	1										1
21	*Procandida tropicalis*	+		1									1
22	*Procandida sp., Pr. grubyi**.	+	1										1
23	*Pseudohansenula sp.*	—		1									1
24	*Rhodotorula rubra*	—						1					1
25	*Saccharomyces cerevisiae*	+	1										1
26	*Torulopsis glabrata*	—						2				1	3
27	*Torulopsis sp., T. westerdijkii**	—				1							1
28	*Trichosporon undulatum*.....	+					1						1
	Total		11	9	8	9	2	18	1	1	3	1	63

*Strains marked with asterisk are described in a separate paper [12].

pus, 1; urine, 1; materials received on culture media, 3. The 63 fungal species isolated from the above materials are listed in Table I.

Of the results the following observation made on 17 patients treated in a sanatorium for pulmonary diseases, is of special interest. Laboratory examination yielded from 7 sputum samples 3 *Candida claussenii*, 1 *Geotrichum candidum*, 1 *Cryptococcus neoformans*, 1 *Procandida majoricensis and* 1 *Procandida grubyi*; from 6 bronchoscopic samples 1 *Candida pseudotropicalis*, 3 *Procandida albicans*, 1 *Candida claussenii* and 1 *Pseudohansenula sp.;* from 4 laryngeal swabs 1 *Candida claussenii*, 1 *Procandida albicans*, 1 *Candida pseudotropicalis* and 1 *Paratorulopsis bánhegyii* strains were isolated.

These findings are noteworthy, since according to our present views the fungi encountered can be interpreted (a) as non-pathogenic agents occupying an intermediate position between saprophytism and parasitism; (b) as primary pathogens; (c) as secondary pathogens. Case (a) can be excluded whenever the strain occurs in a closed cavity or in organs free from fungi in the healthy individual. Differentiation between (b) and (c) is important only for elucidating the case history, but is indifferent as to therapy, since both cases call for antifungal treatment.

Fungi isolated from sputum are important mainly for floristic purposes, as their association with the disease, although not excluded, cannot be confirmed for certain. On the other hand, the bronchi of normal persons are always free from fungi [9], and contamination of bronchoscopic samples and laryngeal swabs from the oral mucosa is unlikely. Therefore isolation of fungi from such materials is to be evaluated positively especially because these agents are liable to modify the course of the disease and thus treatment should be aimed not exclusively against the organism causing the primary infection [10]. It should be noted that at the Leipzig Congress on Fungous Diseases VANBREUSEGHEM [11] stressed that the presence of yeast-like organisms at sites free from fungi in normal persons should be evaluated positively. Although they by themselves may not be responsible for the pathological conditions, fungi may contribute to their development.

We have found yeasts to occur in several patients who were displaying tuberculosis-like diseases, but were always negative for *M. tuberculosis*. Of 17 fungal strains isolated from such patients, 13 were pathogenic in mice. Thus it may be presumed that these organisms were, at least partly, responsible for the pathological conditions. A good example confirming this consideration was that of a patient, who had never been proved to harbour tubercle bacterium, but excreted *Candida claussenii* and *Geotrichum candidum* in his sputum. At necropsy *Candida claussenii* but no *M. tuberculosis* was isolated.

The species distribution of the isolated fungi was very variable. Only *Candida claussenii* and *Procandida albicans* were encountered with some frequency (11.1 and 22.2 per cent, respectively).

Some unclassified strains, which, in our opinion, correspond to new species, will be dealt with in a separate paper [12].

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BIOCHEMICAL STUDIES ON STREPTOMYCES AUREOFACIENS

IV. STUDIES ON THE BIOSYNTHESIS OF CHLORTETRACYCLINE

By

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Summary. By the use of C^{14} and Cl^{36} labelled compounds, it has been shown that, prior to incorporation into chlortetracycline, the chlorine of organic substances is converted into chloride. This observation holds also true for chloropropandiol, which, however, in fermentations carried out with the tetracycline-producing strain CDSD-314, was not utilized for building up chlortetracycline. It has, therefore, been concluded that chloropropandiol does not act as a direct precursor in chlortetracycline biosynthesis.

Both strains used (B-28 and CDSD-314) were able to convert the chlorine of organic compounds into chloride.

Our findings have supported the assumption that 2,5-dimercapto-1,3,4-thiadiazole inhibits the first, oxidative stage of biochemical chlorination. The genetic block of chlorination in the tetracycline-producing strain CDSD-314 is probably restricted only to the first, oxidative stage. As in chlortetracycline biosynthesis no residual reaction was revealed with strain CDSD-314, there are not necessarily two or more genetic routes of biochemical chlorination throughout all stages of the process.

A difference in the incorporation between carbon 1 and 2 of acetate indicated that carbon 2 might take part as C_1 unit in the biosynthesis of the tetracycline molecule.

Our studies on the chlorination mechanism in *Streptomyces aureofaciens* have shown that chlorine of chlorinated fatty acids is utilized for the biosynthesis of chlortetracycline (CTC) [1, 2]. Similarly, SEKIZAWA showed that chloropropandiols can also be used as chlorine sources for CTC synthesis. In the presence of these substances oxytetracycline-producing organisms synthesize CTC in addition to oxytetracycline. Known chlorination inhibitors exert no antagonistic action on these processes [3—5]. On the basis of these findings SEKIZAWA assumed that chloropropandiols are directly utilized for CTC production [6].

In the present studies we have examined some steps of CTC biosynthesis. We attempted to locate the site of the inhibitory action on enzymatic processes of chlorination of 2,5-dimercapto-1,3,4-thiadiazole and the site of the genetic block in the tetracycline-producing *Streptomyces aureofaciens* strain CDSD-314.

Materials and methods

The present studies were carried out under the experimental conditions reported previously [8], with shaken synthetic medium cultures of *Streptomyces aureofaciens* strains B-28 [8] and CDSD-314 [9]. The ingredients of the synthetic medium used in these experiments were: starch, 3.5%; NH_4NO_3, 0.8%; KNO_3, 0.2%; glutamic acid, 0.1%; $MgSO_4\ 7H_2O$, 0.1%; $CaCO_3$, 0.6%; KH_2PO_4, 0.06%; $MnSO_4$, 0.003%; $ZnSO_4$, 0.01%; $FeSO_4$, 0.01%; palm oil, 0.3%; pH 6.2—6.4.

In addition to commercial substances the following isotopes obtained from the Institute of Isotopes, Hungarian Atomic Energy Commission, Budapest, were used: $NaCl^{36}$, chloropropandiol-Cl^{36}, sodium acetate-1-C^{14}, sodium acetate-2-C^{14}, sodium monochloroacetate-1-C^{14},and sodium monochloroacetate-2-C^{14}.

The isotopes were added to the cultures under aseptic conditions as described in the experimental part of this paper. Radioactivity measurements were performed in 72-hour cultures as follows. The fermentation medium was adjusted with 5 *N* HCl to pH 2.0 and filtered; the filtrate was saturated with NaCl and tetracyclines were extracted with n-butanol. Of the extracts 0.2—0.3 ml aliquots were gently evaporated to dryness on aluminium planchets. The activity of the obtained fine layer was measured. The total tetracycline content of butanol extracts was between 8000 and 12,000 μg/ml. Radioactive counts for 1 mg of total tetracycline were calculated from the above data and from the amount of the evaporated extract.

For determining the residual $NaCl^{36}$, the fermentation medium was acidified with HNO_3. After filtration chloride ions were precipitated with $AgNO_3$ and separated by a subsequent filtration. The solution was then saturated with NaCl and the tetracyclines were extracted with butanol. The radioactivity of the butanol extract was similar to extracts prepared in the conventional manner. Then the weight and radioactivity of the obtained AgCl precipitate were measured. It should be noted that prior to precipitation with $AgNO_3$, known amounts of chloride were added to the acidified fermentation media, in order to obtain a precipitate 250—350 mg in weight.

Radioactivity determinations were performed with an end window Geiger-Müller counter or with the Orion-Friesecke-Höpfner counter supplied with a scintillation head.

In order to separate tetracyclines for a qualitative checking of biosynthesis, the butanol extract was paper-chromatographed [8, 10].

Results

In view of the results obtained in our previous studies on the biosynthesis of CTC [11], in the first part of the present experiments *Str. aureofaciens* strains B-28 and CDSD-314 were compared for CTC biosynthesis. As a chlorine source $NaCl^{36}$ was used. CTC production was examined also in the presence of 2,5-dimercapto-1,3,4-thiadiazole (TDA) and Cu^{++} ions.

The results are summarized in Table I. In all experiments $NaCl^{36}$ at a concentration of 0.4 μc/80 ml corresponding to 3 *mM* NaCl, was used.

From Table I it is seen that TDA caused a 74 per cent decrease in CTC production by strain B-28. The antagonizing effect of copper was also definite:

Table I

Incorporation of chloride into CTC

Strain	Additions	CTC+TC units/ml	Radio-activity c. p. m.	Residual chloride per cent
B—28	—	1080	1512	32
B—28	0.2 *mM* TDA	1040	396	80
B—28	0.2 *mM* TDA + 0.3 *mM* $CuSO_4$	825	1180	38
CDSD—314	—	1370	38	98
CDSD—314	0.2 *mM* TDA	1280	32	96
CDSD—314	0.2 *mM* TDA + 0.3 *mM* $CuSO_4$	1010	36	99
CDSD—314	0.3 *mM* $CuSO_4$	980	38	96
—	—	—	40	97

in the presence of 0.3 *mM* $CuSO_4$ the inhibitory action of TDA decreased to 26 per cent.

Strain CDSD-314 was unable to use chloride for CTC biosynthesis and produced only tetracycline (TC). As it is evident from Table I, the low radioactivity is attributable to a chloride contamination of the butanol extract. It is also clear that TDA exerted no effect on TC biosynthesis by strain CDSD-314 and that $CuSO_4$ did not induce CTC production.The slight increase in radioactivity at higher (1.0 μc/80 ml, equivalent to 7.5 *mM*) $NaCl^{36}$ concentrations, was due to a chloride contamination of the butanol extract.

After butanol extraction the $NaCl^{36}$ content of the residual fermentation fluid was also determined. As calculated from the weight of the AgCl precipitate and from the specific radioactivity, the chloride content of the medium was unaltered after fermentation. Thus it may be concluded that chloride was not utilized for the biosynthesis of CTC.

In subsequent experiments the biosynthesis of CTC from chlorinated fatty acids by strains B-28 and CDSD-314 was investigated. As substrates acetate-1-C^{14}, acetate-2-C^{14}, monochloroacetate-1-C^{14} and monochloroacetate-2-C^{14} were used. All substrates were added to the medium at 1 μc/80 ml concentration prior to starting, or at the 24th hour of, fermentation. The results are summarized in Tables II and III.

It is seen that monochloroacetates were utilized by both strains even in the presence of TDA. Splitting chlorine off monochloroacetate was not antagonized by TDA, and, of course, this inhibitor exerted no influence on the utilization of acetate in the biosynthesis of tetracycline.

Table II

Incorporation of acetate 2-C^{14} into CTC+TC

Strain	Additions	Time of addition hrs.	CTC+TC units/ml	Radioactivity c. p. m.
B—28	Acetate-2-C^{14}	0	1010	2450
B—28	Acetate-2-C^{14}	24	1120	2010
B—28	Monochloroacetate-2-C^{14}	0	1050	3170
B—28	Monochloroacetate-2-C^{14}	24	1090	2750
B—28	Monochloroacetate-2-C^{14} + TDA	0	1030	2910
CDSD—314	Acetate-2-C^{14}	0	1250	2920
CDSD—314	Acetate-2-C^{14}	24	1380	2320
CDSD—314	Monochloroacetate-2-C^{14}	0	1170	3470
CDSD—314	Monochloroacetate-2-C^{14}	24	1310	3010
CDSD—314	Monochloroacetate-2-C^{14} + TDA	0	1200	3380

Table III

Incorporation of acetate 1-C^{14} into CTC+TC

Strain	Additions	Time of addition hrs.	CTC+TC units/ml	Radio-activity c. p. m.
B—28	Acetate-1-C^{14}	0	1270	1350
B—28	Acetate-1-C^{14}	24	1120	1210
B—28	Monochloroacetate-1-C^{14}	0	1130	2060
B—28	Monochloroacetate-1-C^{14}	24	1190	1680
B—28	Monochloroacetate-1-C^{14} + TDA ...	0	1180	2110
CDSD—314	Acetate-1-C^{14}	0	1480	1870
CDSD—314	Acetate-1-C^{14}	24	1340	1650
CDSD—314	Monochloroacetate-1-C^{14}	0	1330	2190
CDSD—314	Monochloroacetate-1-C^{14}	24	1410	1990
CDSD—314	Monochloroacetate-1-C^{14} + TDA ...	0	1310	2120

A comparison of Table II and Table III, which present data of experiments carried out under identical conditions, reveals that from acetates carbon 2 was incorporated into TC in a higher proportion than carbon 1. When acetates were added at the beginning of fermentation, the difference was 57 per cent; when they were added at the 24th hour the difference amounted to 53 per cent.

It should be noted that the two strains differed in the utilization of acetates. On the average, the difference amounted to 16 per cent for carbon 2 and to 13 per cent for carbon 1. Both kinds of acetate were utilized more actively by strain CDSD-314.

If SEKIZAWA's assumption [6] that chloropropandiol is incorporated directly into the CTC molecule, would be correct, CTC should be formed in the presence of this substance by TC-producing strain CDSD-314. The results of our experiments in this respect are summarized in Table IV. At the beginning or at the 24th hour of fermentation 0.5 μc/80 ml (4mg/ml) Cl^{36}-labelled chloropropandiol was added to the culture. TDA, which had been sterilized separately, was added to the medium at a 0.2 *mM* concentration at the start of fermentation.

Table IV shows that CTC-producing strain B-28 utilized chloropropandiol for the biosynthesis of CTC. The inhibiting action of TDA is also evident. On the other hand, the TC-producing strain CDSD-314 was unable to utilize chloropropandiol and formed consequently no CTC. At the end of fermentation radioactive chloride was demonstrated in both cultures, even in fermentations inhibited by TDA.

Table IV

Effect of chloropropandiol on the biosynthesis of CTC

Strain	Additions	Time of addition hrs.	CTC+TC units/ml	CTC per cent	Chloride at end of fermentation
B—28	—	—	1140	2	—
B—28	Chloropropandiol	0	870	100	+
B—28	Chloropropandiol	24	790	85	+
B—28	Chloropropandiol + TDA	0	1070	37	+
CDSD—314	—	—	1490	0	—
CDSD—314	Chloropropandiol	0	960	0	+
CDSD—314	Chloropropandiol	24	730	0	+
CDSD—314	Chloropropandiol + TDA	0	1030	0	+

Discussion

In examining chlorination in the biosynthesis of CTC, we have attempted to show the site of the inhibitory action exerted by TDA. The presence of chloride at the end of TDA-inhibited fermentations confirms the opinion [2—6] that TDA-type inhibitors act in the first, oxidative, stage of chlorination. This is also indicated by the finding that the action of TDA is specifically reversed by copper [12].

The biosynthesis of CTC in strain CDSD-314 is inhibited probably in the first stage of the chlorination process. The lack of CTC production is a result of genetic alteration which occurred during the procedure used for the selection of the strain [9]. Chloride was present at the end of fermentations performed with strain CDSD-314, as well as at the end of TDA-inhibited fermentations. There is, however, a considerable and principal difference between inhibition by TDA and the genetic block in strain CDSD-314. The genetic block is irreversible and is not influenced by copper and other substances.

From Table I it is clear that no residual reaction occurs with strain CDSD-314. SEKIZAWA has assumed that the chlorination process generally involves a residual reaction [6]. In his opinion chlorination may proceed in at least different genetic pathways. Similarly, DOERSCHUK *et al.*, who classified *S. aureofaciens* strains on the basis of halide metabolism [13], assumed the presence of that residual reaction in mutants of these microorganisms. In view of the present experiments, this classification should be extended. Strain CDSD-314 does not belong to group II, and cannot, of course, be included in group I. Thus it should be classified into a third group containing cultures unable to utilize chloride for the biosynthesis of CTC. Thus, according to this system, strains belonging to group I utilize chloride independently of its

concentration (Cl-scavengers). The degree of chloride utilization for CTC synthesis by cultures falling into group II depends on the concentration (Cl-non-scavengers). Finally, group III includes strains unable to use chloride in any concentration (Cl-ignoring cultures).

In our opinion the appearance of the residual reaction, in other words, the biosynthesis of relatively small amounts of CTC, greatly depends on the site of the genetic block. We assume that in the first stage of chlorination there are no alternative manners of CTC biosynthesis; it cannot, however, be excluded that in further steps the chlorination process may divide into two more different pathways.

It has been shown in our experiments that TDA does not inhibit the entry into the tetracycline molecule of either carbon 2 or carbon 1 of monochloroacetate (Tables II and III). This finding and the observation that chloride is present at the end of TDA-inhibited fermentations (Table I) indicate that in the first stage of chlorination the chlorine atom is converted into chloride ion, which, in case of strain B-28 and in the absence of TDA, is then incorporated into the CTC molecule.

As shown in Tables II and III, chlorine is split from monochloroacetate by both strains. This finding indicates that the enzyme or enzyme system responsible for breaking up the carbon-chlorine bond and thus for the production of chloride ions, is genetically intact in strain CDSD-314.

Our experiments invariably indicated that the chlorine present in chloropropandiol, similarly to that of monochloroacetate, is converted by strain B-28 into chloride prior to incorporation into CTC (Table IV). After our experiments had been brought to an end [14], GOODMAN *et al.* have published their assumption that the antagonistic action of chloropropandiol on chlorination inhibitors described by SEKIZAWA was attributable to the alkylation of inhibitors by chloropropandiol [15]. This assumption has been confirmed by the present findings, since when sterilized separately and added to the culture at the start of fermentation, TDA was found to exert an inhibitory action (Table IV). Pure TC demonstrated at the end of fermentation by strain CDSD-314, indicated that chloropropandiol cannot be considered a direct precursor in CTC biosynthesis.

From our results it seems that in the chlorination process two different stages might be distinguished. In the first step chlorine is oxidized probably to chlorinium ion, similarly to the reaction observed in *Caldariomyces fumago* by SHAW *et al.* [16]. As we have shown previously [12], a form of chlorine oxidated more than chlorinium, plays no part in CTC biosynthesis. The chlorinium ion, probably bound to an enzyme, reacts in the second stage of chlorination with a common precursor of TC or CTC. The nature or structure of the common precursor is unknown. As revealed in the ingenious experiments of MCCORMICK *et al.* [17], it may be identical with pretetramide (1, 3, 10, 11,12-

pentahydroxy-naphthacene-2-carboxamide) or with 6-methyl-pretetramide, which may be converted into 7-chloro-6-dimethyl-tetracycline or CTC by a suitable strain.

TDA inhibits only the first, oxidative stage, and leaves the second stage unaffected. Although the presented experiments do not exclude the possibility that the genetic block in strain CDSD-314 affects both stages, it seems probable that it is restricted to the oxidative stage.

The difference in the incorporation of carbon 2 and carbon 1 of acetates sheds some light on the biogenesis of the tetracycline molecule. It is generally believed ("polyacetate" hypothesis) [18—21], that the corresponding carbon atoms are incorporated into the tetracycline molecule at equal proportions. Using a non-synthetic medium MILLER *et al.* found that carbon 2 and carbon 1 were utilized at an almost equal ratio [22]. On the basis of the incorporation percentage of acetate-2-C^{14} and the radioactivity of decomposition products, SNELL *et al.* concluded that oxytetracycline-producing *Streptomyces rimosus* synthesized the antibiotic from C_2 units and glutamic acid [23]. Our results also show that this type of biosynthesis plays an important part in the biogenesis of tetracycline. The obtained significant difference indicates, however, that, at least in glycine and methionine deficient synthetic medium, acetate carbon 2 may act in the biosynthesis of tetracycline also as C_1 unit. The role of glycine and methionine in the biosynthesis of dimethylamino and 6-methyl groups has been demonstrated [22—25]. It may be assumed that in our experiments acetate carbon 2, perhaps through a glycine synthesis, contributed to the biosynthesis of tetracyclines as a C_1 unit. For a proper interpretation of the results further experiments are desirable.

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COMPARATIVE EXAMINATION OF CHRONIC TYPHOID CARRIERS WITH IMMUNOFLUORESCENT AND CULTURAL METHODS

By

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Summary. Faecal samples from 200 typhoid carriers have been examined in order to compare immunofluorescent tracing with the conventional enrichment cultivation technique. The presence of *S. typhi* was confirmed in 84 samples (42 per cent) with cultivation and in 139 samples (69.5 per cent) with immunofluorescent detection. Only 2 specimens giving positive cultures were negative with the immunofluorescent method which as a whole, increased the number of positive results by 27.5 per cent. Between immunofluorescent positivity obtained within 1 hour and cultivation there was a 95 per cent agreement. The specific fluorescence of *S. typhi* was highly increased when the faecal samples were alkalized.

The detection of chronic carriers, who may be dangerous sources of endemic outbreaks, presents an important epidemiological problem all over the world. The greatly diminished morbidity from typhoid fever is a result of the registration of carriers and systematic laboratory control of contacts and transient carriers.

According to Bakács, between the two world wars the average annual number of typhoid cases in Hungary was 12,000 with 600 deaths. Until 1960 this number decreased to 600, with less than 10 fatal cases [1]. The general opinion is that minor outbreaks occurring at present originate mainly from carriers, especially from those employed in the food and catering trades.

In Hungary 480 and 438 typhoid cases occurred in 1961 and in 1962, respectively [2]. Therefore the elaboration of new methods and their widespread application in combination with conventional procedures for the improved detection of carriers, would be desirable.

Our previous investigations have shown that the immunofluorescent method allows a very sensitive and specific tracing of low numbers of bacteria. This technique has, therefore, been employed for the examination of chronic typhoid carriers.

Materials and methods

Cultivation methods. Parallel with culturing on plates, the samples were inoculated into selenite enrichment medium. Cultures grown in the latter were seeded after 24 hours onto the usual media. Suspected *S. typhi* colonies were examined for all differential diagnostic characters.

Immunofluorescent tracing. The supernatants of diluted and sedimented faecal samples, which had been adjusted to pH 8 with *N* NaOH, were used for preparing smears. Indirect immunofluorescent examinations were performed as described by Coons and Kaplan [3]. Salmonella O 9.12 and Vi sera prepared in rabbits served as specific immune sera. Rabbit anti-

globulin was conjugated with fluorescein isothiocyanate. For checking aspecific staining control smears were treated with *Sh. sonnei* immune serum.

The smears were examined under a Leitz Model Ortholux II fluorescence microscope at a magnification of 400, in blue light.

Results

The 42 per cent positivity obtained with conventional cultivation was in accordance with the average annual positivity, which amounted at the Department of Bacteriology, State Institute of Hygiene, to 49 and 45.1 per cent in the years 1962 and 1963, respectively.

The examinations were based primarily on the tracing of Vi antigen. The smears were also checked with group serum O 9.12. The relatively low titre of Vi sera *per se* may be responsible for a less bright fluorescence. Improvement of the specific fluorescence yielded by low titre sera was attempted in numerous model experiments. It has been found that immune sera conjugated with flourescein isothiocyanate give a more intensive fluorescence at pH 8 and that alkalization does not influence the development of a strong binding between antigen and antibody. A further advantage of this method is that the conjugate containing no free dye molecules, exerts weak aspecific reactions with other faecal constituents, and therefore a less definite aspecific fluorescence appears after washing.

By the use of this modification, the pathogenic bacteria were well differentiated from other enteric organisms and amorphous faecal constituents exhibiting a characteristic autofluorescence. A comparison of cultural and immunofluorescent examination of faecal samples from chronic typhoid carriers selected at random, is presented in Table I.

Table I

Comparison of the result of cultural and immunofluorescent examination of faecal samples

			Immunofluorescent tracing				Total	
			Positive		Negative			
			Number	Per cent	Number	Per cent	Number	Per cent
Cultivation	Positive	Number	82		2		84	
		Per cent		41		1		42
	Negative	Number	57		59		116	
		Per cent		28.5		29.5		58
	Total	Number	139		61		200	
		Per cent		69.5		30.5		100

From Table I it is seen that immunofluorescent tracing failed to reveal the presence of *S. typhi* only in 2 samples which were positive on cultivation. This result is explained by the fact that when only Vi serum is used, Vi minus strains, which occur only exceptionally, cannot be detected by the immunofluorescent method. The immunofluorescent method gave positive results with 57 samples which were negative on cultivation. It should be noted that the microscopic immunofluorescent picture allows a good estimation of the expectable cultural results. It has been found that cultivation was always positive when at a magnification of 250—400 fluorescing organisms were visible in very large numbers.

Discussion

As a result of systematic detection of carriers, preventive vaccinations and improvement of hygienic conditions, the typhoid fever morbidity shows a continuously decreasing tendency. Undetected carriers, however, present a further potential danger for their environment, and, if working in the food or catering trades, also for other persons. This consideration has been confirmed by widespread screening and by laboratory examinations attempting to use the determination of the rise in phage titre for the final eradication of typhoid fever.

Fromme *et al.* [4] reported the results of surveys made on five million subjects between 1950 and 1957. After the patients' fever had ceased 5 consecutive faecal and one duodenal juice cultures were examined. After recovery, 4 or 5 examinations revealed the pathogenic agent in 21 per cent of the patients. The 6th examination revealed 14 per cent carriers among the discharged patients.

Meyer-Oschatz [5] examined the role of milk in endemic typhoid infections. Between 1947 and 1958 21 milk-borne typhoid or paratyphoid outbreaks affecting 4513 persons were analyzed. Of the patients involved, 2711 were infected with *S. typhi*. Of the 21 outbreaks 19 were due to milk infected by chronic carriers.

Koretzkaya and Yefimova [6] found at necropsy 3.5 per cent typhoid—paratyphoid carriers among persons died from other diseases.

According to Tomcsik [7], Zhdanov [8] and Walter and Wachs [9,10], 2 to 6 per cent of typhoid patients remain carriers.

These data explain clearly why attempts have been made at identifying enteric pathogens with the highly sensitive and specific immunofluorescent method. Thomason, Cherry and Moody [11] and Thomason, Cherry and Edwards [12] have made use of it for the examination of faecal samples from normal persons, typhoid carriers and patients suffering from acute gastroen-

teritis. GECK *et al.* [13] compared the diagnostic efficacy of cultivation, immunofluorescent tracing and passive haemagglutination in patients hospitalized with enterocolitis. They found that, as compared to cultivation, the immunofluorescent method increased the number of Shigella positive results almost three times.

The last quoted finding has been confirmed by the present investigations, which have shown that by the use of the modified technique for preparing smears, an intensive specific fluorescence is obtained even with low titre sera. The 69.5 per cent positivity revealed by immunofluorescent tracing as compared to the 42 per cent positivity of cultivation, calls attention to the sensitivity of this method, rather than to its rapidity. Considering that carriers are sometimes periodic excreters and, depending on their immunological state, they often harbour *S. typhi* in low counts, it seems probable that immunofluorescent method combined with the conventional cultivation technique and the determination of the rise in phage titre is suitable for the detection of typhoid carriers.

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STUDIES ON THE HUMAN INTESTINAL FLORA

II. ALTERATIONS IN THE INTESTINAL FLORA OF PATIENTS TREATED WITH ANTIBIOTICS

By

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Summary. (i) Chloramphenicol, especially when given parenterally, exerted a slight effect on the intestinal flora. Chlortetracycline caused a more definite injury mainly of the normal anaerobic constituents. The most serious damage to the aerobic and anaerobic flora was noted after erythromycin treatment.

(ii) Simultaneously with the reduction of some constituents or of the entire normal flora, certain normal *(Str. faecalis)* or accidental organisms (clostridia, staphylococci) became occasionally predominant, without any definite association.

(iii) It has been shown that *Staph. aureus* strains occurring in faecal samples originated from the respiratory tract.

(iv) Although several serious alterations were revealed in the intestinal flora of the examined patients, clinical complications due to the applied antibiotics had never developed. It has, therefore, been concluded that a reduction of normal constituents or an increase in the number of other microorganisms are by themselves not responsible for complications occurring in the course of antibiotic therapy.

(v) Data obtained in animal experiments as to the presence or absence of antagonism among some constituents of the intestinal flora, have been confirmed.

Observations made on the normal intestinal flora [1] have yielded a basis for an adequate theoretical and practical evaluation of bacteriological alterations revealed in antibiotic-treated patients. The present examinations were carried out on patients involved in our previous study of the normal intestinal flora.

Materials and methods

Taking of faecal samples, cultivation technique, media, testing of antibiotic resistance and statistical methods have been described previously [1].

Drugs. Chloramphenicol (Chlorocid, United Drug Co., Budapest); chlortetracycline (Xanthomycin, United Drug Co., Budapest); erythromycin (Erythrocin, Abbot Laboratories Ltd., London), were prescribed on the basis of clinical indication.

Daily examination of the faecal flora was commenced 2 to 4 days before the administration of antibiotics had begun. Specimens were taken throughout treatment. The examinations were continued until in the post-therapeutic period normalization of the flora had become evident.

Estimation of alterations in intestinal flora. The decrease in bacterial count was regarded as *serious* when the number of bacteria decreased by 3 to 4 exponents, this condition lasted for more than 6 days and normalization occurred only after treatment had been discontinued. A *moderate* change was characterized by reduction by 2 to 3 exponents in bacterial count for 3 to 6 days and complete normalization during treatment. A *slight* change was considered to occur when the decrease did not exceed 2 exponents, lasted for only 1 to 3 days and the flora had normalized during treatment.

A given organism was regarded to be *overgrown* when its count became higher by at least 2 exponents.

Results

(1) *Effect of chloramphenicol injection.* The effect of 10 to 16 g chloramphenicol administered parenterally during 5 to 8 day periods to each of 11 patients, is presented in Table I.

It is seen that, presumably owing to the antibiotic concentration being low in the intestinal tract, parenteral chloramphenicol exerted a slight influence on the constitution of the normal flora. In 4 cases the number of aerobic, in 1 case of anaerobic bacteria decreased slightly. In the remaining 6 patients no altera-

Table I

Effect of chloramphenicol injection on the intestinal flora
(11 patients each treated with 10—16 g chloramphenicol during 5—8 days)

	Degree of injury			
	Nil	Slight	Moderate	Serious
Aerobic flora	1	4	—	—
Anaerobic flora	4	1	—	—
Anaerobic-aerobic flora	6	—	—	—
	Unchanged		Overgrown	
E. coli	11		—	
Kl. pneumoniae	10		1	
Str. faecalis	10		1	
Staph. aureus	9		2	
Clostridium	11		—	

tions were observed. Unimportant increases in Klebsiella count occurred in 1, in *Str. faecalis* count in 1 and in staphylococcal count in 2 persons. The slight changes in the normal flora were not associated with clinical complications attributable to the antibiotic.

(2) *Effect of chloramphenicol tablets.* Ten to 16 g chloramphenicol given orally to each of 10 patients during 5 to 8 day periods yielded the results summarized in Table II.

Table II indicates that a serious injury to the normal flora had not occurred. A moderate simultaneous damage of the aerobic and anaerobic flora occurred in one case only. In 5 of the 10 patients no alteration in the flora was observed. In some cases insignificant increases in *E. coli*, *Kl. pneumoniae*, *Staph. aureus* or Clostridium counts were revealed. Changes in the bacterial flora were not associated with clinical disturbances.

Table II

Effect of chloramphenicol tablets on the intestinal flora

(10 patients, each treated with 10—16 g chloramphenicol during 5—8 days)

	Degree of injury			
	Nil	Slight	Moderate	Serious
Aerobic flora	1	—	1	—
Anaerobic flora	1	1	—	—
Anaerobic-aerobic flora	5	2	1	—

	Unchanged	Overgrown
E. coli	9	1
Kl. pneumoniae	9	1
Str. faecalis	10	—
Staph. aureus	9	1
Clostridium	9	1

It should be noted that routinely performed haematological examinations revealed no change in the blood counts in any of the 21 patients receiving chloramphenicol treatment.

(3) *Effect of chlortetracycline tables.* The effect of 10 to 16 g chlortetracycline administered orally to each of 10 patients during 5 to 11 day periods is shown in Table III.

Table III

Effect of chlortetracycline tablets on the intestinal flora

(10 patients each treated with 10—16 g chlortetracycline during 5—11 days)

	Degree of injury			
	Nil	Slight	Moderate	Serious
Aerobic flora	3	1	—	—
Anaerobic flora	—	—	1	3
Aerobic-anaerobic flora	4	1	1	—

	Unchanged	Overgrown
E. coli	9	1
Kl. pneumoniae	9	1
Str. faecalis	5	5
Staph. aureus	8	2
Clostridium	8	2

Chlortetracycline was damaging mainly the anaerobic constituents. The aerobic flora decreased moderately in one and slightly in another patient. The anaerobic flora was reduced considerably in 3, moderately in 2 persons. In 4 chlortetracycline-treated patients the intestinal flora was unaltered. The increase in the number of various bacteria *(E. coli*, 1 case; Klebsiella, 1 case; Staphylococcus, 2 cases; *Str. faecalis*, 5 cases) was more common. In spite of these changes, no clinical complications developed.

(4) *Effect of erythromycin tablets.* Table IV presents the effect of 10 to 20 g erythromycin given orally to each of 10 patients during 5 to 12 day periods.

The intestinal flora of all patients receiving erythromycin suffered more or less definite alterations. In one patient a slight reduction of the anaerobic flora and a moderate decrease in the aerobic flora occurred. In one further patient the number of both anaerobic and aerobic organisms decreased moderately. In 8 cases, except for *Str. faecalis*, an important decrease of the total flora occurred. Erythromycin-resistant *Str. faecalis* proliferated in 6 cases. In 3 patients Staphylococcus appeared in larger numbers. Even the important bacteriological alterations were not associated with clinical disorders.

Table IV

Effect of erythromycin tablets on the intestinal flora

(10 patients, each treated with 10—20 g erythromycin during 5—12 days)

	Degree of injury			
	Nil	Slight	Moderate	Serious
Aerobic flora	—	—	1	—
Anaerobic flora	—	1	—	—
Aerobic-anaerobic flora	—	—	1	8

	Unchanged	Overgrown
E. coli	10	—
Kl. pneumoniae	10	—
Str. faecalis	4	6
Staph. aureus	7	3
Clostridium	10	—

Summarizing the alterations occurring in the four groups of patients, it may be concluded that the intestinal flora was affected least by parenteral or oral chloramphenicol treatment. A more definite damage, concerning mainly anaerobic bacteria, was observed with chlortetracycline treatment. In the majority of patients erythromycin gave rise to an almost complete elimination

of the normal flora. In spite of all these alterations, not excepting those produced by erythromycin, clinical complications were never observed.

In view of the theoretical and practical importance of the question, the increase in the number of various constituents is discussed in detail. The relevant results are summarized in Table V.

In Table V the data are grouped according to antibiotics and overgrown microorganisms. From among the normal constituents, the organism becoming most frequently predominant was *Str. faecalis* (12 cases). Of the accidental constituents Staphylococcus was the one most frequently appearing in increased counts. The increase of *E. coli* occurred in 2, of *Kl. pneumoniae* in 3 and of

Table V

Members of the intestinal flora occurring in increased numbers during antibiotic treatment

Organisms	Chloramphenicol		Chlortetracycline tablets	Erythromycin tablets
	tablets	injection		
E. coli	1	—	1	—
Kl. pneumoniae	1	1	1	—
Str. faecalis	—	1	5	6
Staph. aureus	1	2	2	3
Clostridium	1	—	2	—
Total	4*	4*	11**,***	9**

* *Staph. aureus* and *Str. faecalis* increased simultaneously
** In 2 cases *Staph. aureus* and *Str. faecalis* increased simultaneously
*** In 1 case *E. coli* and *Str. faecalis* increased simultaneously

Clostridium in 3 cases. The number of the anaerobic members of the normal flora never increased. In view of their small number, cases showing an increase of *E. coli*, *Kl. pneumoniae* and clostridia could not be evaluated. In contrast, analysis of the material displaying increase of *Str. faecalis* and Staphylococcus allowed to draw some conclusions. The data are grouped in Table VI according to alterations in the normal flora.

In the upper part of Table VI cases are presented in which *Str. faecalis* and *Staph. aureus* increased separately or simultaneously. It is clear even without statistical confirmation that no association could be revealed between the increases in the counts for the two organisms ($\chi^2 = 0.833$; $P < 0.50$ and > 0.30). The lower part of Table VI indicates the association between the conditions of the intestinal flora and the increase of *Str. faecalis* or *Staph. aureus*. The absolute or relative increase of *Str. faecalis* occurred simultaneously with the reduction in the number of aerobic or anaerobic constituents, but *Str. faecalis* was present in higher numbers even in cases with no alteration of

Table VI

Association between the increase in Str. faecalis and Staph. aureus counts and changes in the intestinal flora

Simultaneous increase of *Str. faecalis* and *Staph. aureus:* 5 cases
Increased *Str. faecalis* — unchanged *Staph. aureus* counts: 7 cases
Increased *Staph. aureus* — unchanged *Str. faecalis* counts: 3 cases

	Str. faecalis count		Staph. aureus count	
	increased	unchanged	increased	unchanged
E. coli count reduced	—	6	1	5
Anaerobic count reduced	2	7	1	8
E. coli + anaerobic count reduced	6	6	2	10
Normal flora unchanged	4	9	4	9
Homogeneity $-\chi^2_{(3)}$	3.380		0.091	
P	0.50—0.30		> 0.99	

the latter bacteria. Due to the small number of observations and the high standard error, the χ^2 test shows a 30—50 per cent homogeneity only. The same considerations were true for the association between the increase of Staphylococcus and conditions of the intestinal flora. Because of a more "fortunate" standard error, the homogeneity was at the 1 per cent level. It could thus be concluded that no association existed between the increase in the number of cocci and the changes in the intestinal flora.

For confirming these considerations and demonstrating the character of increased counts, two individually analyzed cases are presented (Figs. 1 and 2).

Fig. 1 shows the rise of *Str. faecalis* counts in an intestinal flora damaged by erythromycin. After the flora had normalized, the *Str. faecalis* count remained at the increased level. It is interesting that an erythromycin resistant *Staph. aureus* strain originally present in large numbers should have disappeared during treatment.

In Fig. 2, in addition to the peak coinciding with the lowest *E. coli* count, other rises in the number of staphylococci can also be observed. There was a smaller peak prior to the decrease of *E. coli* count and a definite peak after the normalization of the coli flora.

Staphylococci occurring in faeces may originate from the oral cavity, throat or respiratory tract. With saliva or sputum considerable numbers of staphylococci may enter the intestinal tract, multiply there and become a common member of the faecal flora. In view of this consideration, 6 of our patients harbouring higher numbers of staphylococci, were more extensively examined (Table VII).

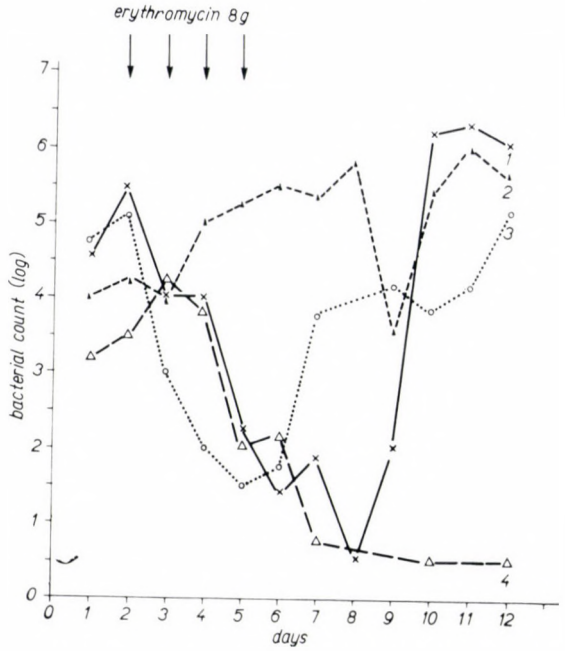

Fig. 1. Alterations in intestinal flora during erythromycin treatment of a patient suffering from suppurative pneumonia. 1: *E. coli* ; 2: *Str. faecalis* ; 3: Normal anaerobes; 4: *Staph. aureus*

Table VII reveals that staphylococci appeared in increased numbers for 1 to 13 days. The maximum count amounted to 0.15—25 per cent of the total

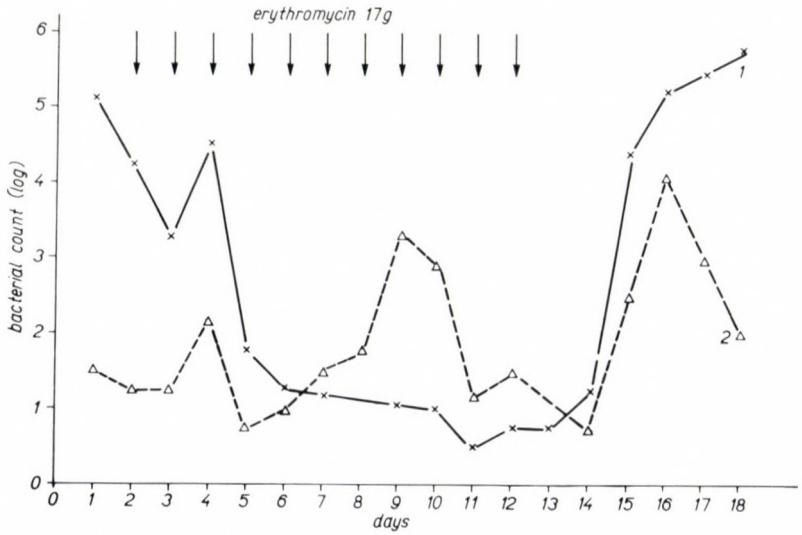

Fig. 2. Alterations in intestinal flora during erythromycin treatment of a patient suffering from rheumatic fever. 1: *E. coli* ; 2: *Staphylococcus aureus*

Table VII

Analysis of cases with important overgrowth of Staph. aureus

Case	Maximum increase as percentage of constituents	Duration of increase, days	Occurrence of *Staph. aureus*		Character of flora	Clinical diagnosis
			in faeces before treatment	in throat or sputum		
1	0.15	2	—	+	normal	Pulmonary abscess
2	0.2	1	—	+	normal	Pneumonia
3	6.0	9	+	+	normal	Suppurative pneumonia
4	0.8	10	+	+	reduction of anaerobes	Septicaemia
5	25.0	13	+	+	reduction of aerobes and anaerobes	Thoracic empyema
6	1.0	3	+	—	normal	Suppurative pneumonia

intestinal flora. In 4 cases, including case 3 with a 6 per cent staphylococcal frequency, the normal flora remained unchanged. It must be noted that all samples had yielded staphylococci prior to antibiotic treatment. Throat or sputum samples were positive for staphylococci in almost every patient, and clinical diagnosis also indicated a staphylococcal infection of the respiratory tract.

Discussion

Our findings concerning the effect of antibiotics on the intestinal flora agree with data in the literature. As revealed by KNOTHE [2], when given parenterally chlortetracycline gave rise to a lower drug level in the intestinal tract and was therefore less injurious to the faecal flora than on oral administration. As noted also by KNOTHE [3] and HAENEL [4], chlortetracycline was particularly harmful for the anaerobic flora. The strong viable count-reducing activity of erythromycin on the normal flora has been observed by TAKIGAMI [5] and ourselves [6] on aerobic bacteria.

In the opinion of several authors, the reduction of the normal flora or its antagonistic action and the increase of certain normal (mainly *Str. faecalis*) or accidental (mainly Staphylococcus) constituents and the impairment of conditions hindering their pathogenic action, are responsible for the complications encountered in the course of antibiotic therapy. In the present study, in agreement with KNOTHE and other investigators, no association has been found between the alteration in the count for normal constituents and the increased occurrence of other microorganisms.

As shown in animal experiments [7], the increase in the number of streptococci cannot be attributed to a reduction in the number of other normal constituents. No antagonism was revealed between *Str. faecalis* and other representatives of the normal flora. In antibiotic treated mice possessing a special flora consisting mainly of *L. bifidus*, DUBOS and SCHAEDLER [8] observed the frequent increase of *E. coli* and *Str. faecalis*. No such occurrence was noted in untreated mice.

In so-called secondary infections, Staphylococcus is believed to play a primary role. Cases of enterocolitis occurring after antibiotic treatment were first described by KRAMER [9] in 1948. Subsequently this problem has been investigated by several authors. The aetiology of this condition is difficult to understand, on the one hand because staphylococci have been isolated in equal proportions from patients with and without enterocolitis [10], and, on the other, as clinically well-defined cases of postantibiotic enteritis may yield negative bacteriological findings [11, 12]. It is stressed by PETTET [13] that the precisely definable condition, pseudomembraneous enterocolitis, well-known long before the era of antibiotics, is not more frequent now than it was in the past. KNOTHE [3] is of the same opinion.

Our data have indicated no association between alterations in the intestinal flora and the increase of staphylococcal count. In contrast, the respiratory origin of the staphylococcal strains has been confirmed. Similar observations were made by MCFARLAN *et al.* [14] as well as by MARTIN [15].

Our present knowledge of the problem does not allow to draw conclusions as to the role of antibiotics or to the significance of secondary infections. The opinion that even drastic and durable injuries of the normal flora and the appearance and increase of "undesirable" constituents are by themselves not responsible for clinical complications, seems justified on the basis of the finding that alterations in the faecal flora frequently occur without clinical symptoms.

Finally, the diagnostic difficulties should be pointed out. The quantitative method of analysis of the intestinal flora described in the present paper or other similar techniques are not suitable for routine purposes. The quantitative methods are useful only for determining the presence of a significant or less significant alteration of the flora, but give no information as to whether clinical complications can or cannot be expected during treatment and whether the increased occurrence of some organisms means in reality a superinfection. From the practical standpoint, the present results only indicate that alterations in the normal flora should be interpreted cautiously.

Acknowledgement. We are indebted to United Drug Co., Budapest, for a supply of Chlorocid injections and Xanthomycin tablets.

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IMPLANTATION ANTAGONISM BETWEEN E. COLI AND NON-PATHOGENIC OR FACULTATIVELY PATHOGENIC ENTERIC BACTERIA

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Summary. (i) No implantation antagonism has been revealed among the genera *E. coli* and Klebsiella and the tribe Proteae or within Klebsiella or the Proteae.

(ii) The antagonism observed between *E. coli* and Klebsiella and within Klebsiella differs in character from implantation antagonism.

(iii) When given orally to mice, Pseudomonas and Staphylococcus could be implanted neither in the presence nor in the absence of the normal flora.

(iv) Neither implantation or other kind of antagonism nor definite association was found between *E. coli* and normal, constant members of the intestinal flora *(L. bifidus*, Bacteroides. *Str. faecalis)*.

(v) As to Klebsiella and Proteus, the lack of implantation antagonism, the limited degree of excretion and the finding that the latter can be mechanically eliminated from the intestines indicate that there is no definite connection between these organisms and the intestinal mucosa. Thus it has been concluded that the tribe Proteae and the genus Klebsiella are accidental members of the intestinal flora.

In a previous paper [1] studies in mice of the antagonism within the genus *E. coli* have been described. It has been found that, as to colonization in the intestinal tract, there is an absolute antagonism among *E. coli* strains. All originally present *E. coli* strains inhibit the settling of other *E. coli* cultures entering by chance or by artificial inoculation. The new strains can be recovered from the intestinal tract for 1 week at most. The phenomenon is independent from diffusible antibacterial factors and immunity and has been observed within the same serotype. It has been concluded that a close connection develops between the resident *E. coli* strain and the intestinal mucosa and thus the original inhabitant "interferes" with new, secondarily entering "foreign" *E. coli* cultures.

Data in the literature [2, 3, 4, 5, 6, 7] and our subsequent experiments seemed to confirm this hypothesis. The present paper gives an account of studies concerning the antagonism between *E. coli* and non-pathogenic or facultatively pathogenic bacteria (Klebsiella, Proteus, Pseudomonas and Staphylococcus) frequently encountered in the intestinal flora.

Materials and methods

Cultures. E. coli. O111:B4 : H2 "1194", *P. morganii* Ola,d "702", *P. morganii* Ola,d "554", *P. morganii* 023 "637", *P. vulgaris* 014, a. b "Kauffmann", *P. vulgaris* "128", P. *rettgeri* "455", *P.mirabilis* "988", *P. inconstans* "41", *P. rettgeri* Lac$^+$ "il 60", *P. mirabilis* Lac$^+$

"ml 1", *P. vulgaris* Lac+ "Vl 50", *P. inconstans* Lac+ "Pil 11", *Kl. pneumoniae* 01 "17", *Kl. pneumoniae* 03 "45".

Lactose-fermenting (Lac+) Proteus strains were kindly supplied by Dr. V. L. SUTTER, [9] Veterans' Administration Center, Los Angeles, Calif., USA.

Mutants resistant to 1000—5000 μg streptomycin were selected from each strain by the gradient plate method [8].

Experimental animals. Most of the examinations were carried out in albino mice weighing 20 to 24 g. The animals were kept separately in paper-covered sterile glass cylinders containing sterilized litter. The cylinders were placed in groups in previously disinfected, well-closing boxes. The animals were fed on a sterilized wet mixture consisting of bread and oats.

Chicks free from enteric flora were hatched from eggs which had been thoroughly rubbed with tincture of iodine and placed in sterile glass cylinders containing sterile sand.

Streptomycin treatment. Each mouse received doses of 50 mg streptomycin in 0.5 ml volume daily for 2 days by means of an S-shaped metal catheter.

Oral infection was carried out with suspensions washed off 18—24 hour agar slants, as described for streptomycin treatment.

Cultivation of faecal specimens. Rectal samples were taken by means of a loop moistened with saline. Enterobacteriaceae were cultivated on Endo agar, staphylococci on blood agar and anaerobes on BIERKOWSKI's medium [10].

Bleeding of mice was performed by the method of TILGNER and METZKE [11] through the retrobulbar venous plexus by use of a capillary. In this manner 0.5 to 1.5 ml blood was obtained from each animal. The sera were stored at — 20 °C.

Washing of the intestinal tract and preparation of clutch cultures. The intestinal contents were removed by careful pressing, the intestinal tract was flowed through with abundant amounts of saline, the intestine was blown up, cut into 8—10 cm portions, ripped open and fixed onto handled metal plates somewhat smaller in size than the diameter of a Petri dish. The fixed intestine was then pressed against the surface of an Endo plate. After incubation the developing colonies were identified. The clutch cultivation method presented several technical difficulties and errors and thus only part of the experiments could be evaluated.

Passive mouse protection test. Dilutions representing 0.05, 0.005 and 0.0005 ml serum were injected subcutaneously into groups of 5 mice each. The challenging dose was administered 18—24 hours later, by injecting intraperitoneally approximately 500 LD_{50} amounts of 18—24 hour agar slant cultures of the organism. The challenging dose was titrated in preliminary experiments. The infecting dose for unprotected mice was titrated in parallel virulence tests. Observation lasted for 3 days and the results were evaluated by considering the number of animals surviving the 3 day period. LD_{50} and ED_{50} values were estimated as described by KÄRBER [12].

Results

In the present experiments the method applied earlier [1] has been used. The normal flora was eliminated by streptomycin treatment and its exogenic restitution was prevented by keeping the animals under sterile conditions. Then, by oral introduction of one given strain *(E. coli* or other) a "monotypic" flora was established. The antagonizing activity of this culture against organisms subsequently introduced via the alimentary tract was then investigated.

The experiments have been focussed mainly on Klebsiella and Proteae, which are frequently encountered in the intestinal tract and stand systematically close to the Escherichia group.

(1) *Implantation antagonism between E. coli and the tribe Proteae*

After streptomycin treatment an artificial *E. coli* O111 : B4 : H2 flora was established in mice. The mice were checked for the exclusive excretion of this organism, then 5 days later groups containing 12 animals each were superinfected with various genera of the tribe Proteae *(P. vulgaris, P. mirabilis, P. morganii, P. rettgeri* and *P. inconstans).*

From Table I it is seen that, parallel with the unchanged excretion of the *E. coli* strain, the Proteus cultures were also harboured by an overwhelming majority of the animals until the end of the observation period.

Table I

Implantation antagonism between E. coli and Proteae

Artificial *E. coli* O111 : B4 : H2 flora superinfected with various genera of the tribe Proteae

Strain used for superinfection	Observation period, days			
	2	7	13	17
P. vulgaris	6/12*	9/12	12/12	12/12
P. mirabilis	11/12	12/12	12/12	11/12
P. morganii	12/12	12/12	12/12	12/12
P. rettgeri	12/12	11/12	12/12	9/12
P. inconstans	12/12	12/12	12/12	10/12

* Numerator: number of mice excreting Proteus strains; denominator: number of infected mice

N. B. The artificially implanted *E. coli* was excreted by all mice

In Table II the results of reciprocal infections are demonstrated. After the establishment of a "monotypic" flora with different Proteus strains, these animals 5 days later were superinfected with *E. coli* O111.

Table II

Implantation antagonism between E. coli and Proteae

Artificial Proteus "flora" superinfected with *E. coli* O111 : B4 : H2

Artificial Proteus flora	Observation period, days			
	5	10	14	18
P. vulgaris	12/12*	12/12	12/12	12/12
P. mirabilis	12/12	11/12	12/12	12/12
P. morganii	12/12	12/12	12/12	12/12
P. rettgeri	12/12	12/12	12/12	12/12
P. inconstans	12/12	12/12	12/12	12/12

* Numerator: number of mice excreting *E. coli*; denominator: number of infected mice

N. B. The artificially implanted Proteus strains were excreted by all mice

Similarly to the result of the previous experiment, *E. coli* and the artificially "implanted" Proteus strains were excreted during the observation period (18 days).

The results indicated that no implantation or other kind of antagonism existed between *E. coli* and the tribe Proteae.

Table III

Implantation antagonism within the tribe Proteae

Artificial *P. morganii* O23 flora superinfected with *P. vulgaris* (Lac$^+$), *P. mirabilis* (Lac$^+$), *P. rettgeri* (Lac$^+$) and *P. inconstans* (Lac$^+$)

Observation period, days	Excretion of			
	P. vulgaris Lac$^+$	*P. mirabilis* Lac$^+$	*P. rettgeri* Lac$^+$	*P. inconstans* Lac$^+$
3	5/5*	5/5	5/5	5/5
7	5/5	5/5	5/5	5/5
12	5/5	5/5	5/5	5/5
17	5/5	4/5	5/5	5/5

* Numerator: number of mice excreting the superinfecting Proteus strain; denominator: number of infected mice possessing a *P. morganii* O23 flora

Experiments summarized in Table III demonstrated no antagonism within the tribe Proteae. These experiments were considerably facilitated by the application of the lactose-fermenting Proteus strains of SUTTER [9].

Table III shows that, in addition to *P. morganii* constituting the primary flora, lactose positive *P. vulgaris*, *P. mirabilis*, *P. rettgeri* and *P. inconstans* strains were abundantly excreted.

Table IV

Implantation antagonism within the tribe Proteae

Intrageneric antagonism of *P. morganii*

Observation period, days	*P. morganii* O1 flora		*P. morganii* O23 flora	
	Excretion of		Excretion of	
	O1	O23	O23	O1
4	10/10*	10/10	10/10	10/10
9	10/10	10/10	10/10	10/10
14	10/10	10/10	10/10	10/10

* Numerator: number of mice excreting the organism; denominator: number of infected mice

Finally, the intrageneric antagonism in the tribe Proteae was examined. One of these experiments is presented in Table IV.

In these experiments a "monotypic" flora was established with *P. morganii* Ola,d or with *P. morganii* O23. After cross-infecting with the other strain, it was revealed that during the 24-day observation period both groups of mice excreted both the primary and the secondary organism.

It should be noted that Proteus excretion could be induced with a similar method in mice possessing a normal intestinal flora. However, in the case of both a natural and an artificial *E. coli* flora, the number of Proteus gradually decreased and 2 months after the infection these bacteria had completely disappeared.

Proteus excretion *per se* does not determine the oecological position of this organism. The lack of antagonism against *E. coli* or against other members of its own tribe indicates, however, that no "implantation" occurs, at least not in the sense applied to *E. coli* in our hypothesis. That the survival of Proteus strains in mice is not due to their invasiveness, has been confirmed by protection tests carried out with sera of mice harbouring Proteus strains for longer periods (Table V).

Table V

Titration of protective antibodies in sera of mice excreting Proteus strains for 14 days

(Passive mouse protection test)

Challenging dose, LD_{50} ...	*P. vulgaris*	*P. morganii*	*P. mirabilis*	*P. rettgeri*	*P. inconstans*
	250	500	500	100	100
Protective value of excreter animals' sera in ml	>0.05	~0.05	>0.05	0.028	~0.05
Protective value of normal animals' sera in ml	>0.05	~0.05	>0.05	~0.05	~0.05

Table V shows that no protective titres were encountered in the sera of Proteus-excreting mice.

In order to elucidate the Proteus—host relationship, a mechanical removal of Proteus from the intestinal tract was attempted. The experiments were based on the investigations of SCHAEDLER and DUBOS [13]. Mice possessing a normal intestinal flora were sacrificed and their intestines were removed. Cultures were performed as described under "Materials and methods", after simple removal of the intestinal contents and after washing. The results are shown in Table VI.

Table VI

Recovery of E. coli and P. morganii from the intestinal tract of mice before and after washing

Portion of intestine	Unwashed intestine		Washed intestine	
	E. coli	*P. morganii*	*E. coli*	*P. morganii*
Small intestine, upper portion	—	—	—	—
Small intestine, lower portion	—	+	—	—
Coecum	+	+	+	—
Large intestine	+	+	+	—

Table VI demonstrates the remarkable fact that in contrast to *E. coli*, *P. morganii* can be removed from the intestinal tract by washing.

(2) *Implantation antagonism between the genera E. coli and Klebsiella.* The "monotypic" flora established in mice with *E. coli* and *Kl. pneumoniae* was superinfected with *Kl. pneumoniae* and *E. coli*, respectively. The results are summarized in Tables VII and VIII.

Table VII

Implantation antagonism between E. coli and Kl. pneumoniae

Artificial *E. coli* (O111 : B4 and O19 Lac$^-$) flora superinfected with Klebsiella

Observation period, days	Excretion of			
	E. coli O111	*Kl. pneumoniae*	*E. coli* O19	*Kl. pneumoniae*
3	10/10*	10/10	10/10	10/10
7	10/10	8/10	10/10	4/10
11	10/10	4/10	10/10	3/10
15	10/10	1/10	10/10	2/10
20	10/10	0/10	10/10	0/10
25	10/10	0/10	10/10	0/10

* Numerator: number of excreting mice; denominator: number of infected mice

Table VII shows that in contrast to the persistent carriage of *E. coli* O111 and O19 strains comprising the artificial flora, the excretion of Klebsiella ceased approximately within 2 weeks.

When Klebsiella had been introduced primarily, the multiplication of both *E. coli* (O111 and O19) strains was somewhat inhibited; however, after 1 week the latter bacteria increased in number and were present during the

Table VIII

Implantation antagonism between E. coli and Kl. pneumoniae

Artificial *Kl. pneumoniae* flora superinfected with *E. coli* O111 : B4 and O19 Lac⁻

Observation period, days	Excretion of			
	Kl. pneumoniae	*E. coli* O111	*Kl. pneumoniae*	*E. coli* O19
2	10/10*	4/10	10/10	2/10
5	10/10	6/10	10/10	8/10
8	10/10	10/10	10/10	10/10
12	10/10	10/10	10/10	10/10
15	9/10	10/10	10/10	10/10
18	6/10	10/10	10/10	10/10
21	2/10	10/10	7/10	10/10
24	1/10	10/10	5/10	10/10
27	0/10	10/10	2/10	10/10
30	1/10	10/10	0/10	10/10

* Numerator: number of excreting mice; denominator: number of infected mice

30-day observation period. In contrast, the number of mice excreting Klebsiella decreased. In some animals Klebsiella was present for 24 to 27 days (Table VIII).

It should be noted that this kind of antagonism significantly differs from the implantation antagonism revealed within the genus *E. coli*. *E. coli* exerts an antagonistic action even against the primarily introduced Klebsiella, and the duration of Klebsiella excretion considerably exceeds the 1 week excretion of the secondary strain, which was a maximum in intrageneric coli antagonism.

An antagonism of similar character was observed within the genus Klebsiella (Table IX).

(3) *Implantation antagonism against normal, constant constituents of the intestinal flora.*

(a) *Antagonism between the genera E. coli and Str. faecalis. Str. faecalis* is a constantly encountered member of the intestinal flora of mice. This organism occurred in the faeces of mice in 10^3—10^4 cells/mg count, and comprised 1/10—1/3 part of the *E. coli* count. The experiments revealed no antagonism between the two genera. As demonstrated in Table X, *E. coli* can be implanted in the presence of a *Str. faecalis* flora, and *vice versa* ; *Str. faecalis* does not inhibit the colonization of *E. coli*.

Some unsuccessful experiments were performed in mice and chicks to implant a *Str. faecalis* strain distinguished by antibiotic resistance pattern over the normal *Str. faecalis* flora. In contrast, the implantation of *Str. faecalis*

Table IX

Implantation antagonism within the genus Klebsiella

Artificial *Kl. pneumoniae* O1 flora superinfected with *Kl. pneumoniae* O3

Observation period, days	Excretion of	
	Kl. pneumoniae O1	*Kl. pneumoniae* O3
2	10/10*	6/10
3	10/10	5/10
6	10/10	6/10
9	10/10	5/10
12	9/10	5/10
15	8/10	3/10
19	8/10	1/10
22	7/10	0/10

* Numerator: number of excreting mice; denominator: number of infected mice

into sterile chicks was successful. The present experiments do not allow conclusions as to the existence of an implantation mechanism similar to that revealed in *E. coli*.

(b) *Antagonism between E. coli and normal anaerobic bacteria (L. bifidus, Bacteroides)*. Only some orientative experiments were performed in order to study whether *E. coli* may be implanted in the presence of normal anaerobic constituents. No reciprocal examinations were carried out.

The observations are presented in Table XI.

Table X

Natural Str. faecalis flora superinfected with E. coli O111 : B4 and artificial E. coli O111 : B4 flora superinfected with Str. faecalis

Observation period, days	*Str. faecalis* flora	*E. coli* flora
	Excretion of *E. coli*	Excretion of *Str. faecalis*
3	10/10*	10/10
7	10/10	10/10
12	10/10	10/10
16	10/10	10/10

*Numerator: number of mice excreting *E. coli* or *Str. faecalis;* denominator: number of infected mice

Table XI

Normal anaerobic intestinal flora (L. bifidus and Bacteroides) superinfected with E. coli O111 : B4

Observation period, days	Excretion of	
	E. coli O111	Normal anaerobes
3	5/5*	Unchanged
6	5/5	Unchanged
9	5/5	Unchanged
12	5/5	Unchanged
15	5/5	Unchanged

* Numerator: number of mice excreting *E. coli;* denominator: number of infected mice.

The data indicate that constituents of the normal anaerobic flora do not exert an antagonistic effect on the implantation of *E. coli.*

The behaviour of *Str. faecalis* and anaerobes corresponded to the expected results. Had an antagonism been present, the simultaneous occurrence of different normal constituents would have been unexplainable.

Table XII

Artificial E. coli O111 : B4 : H2 flora superinfected with Ps. aeruginosa

Observation period, days	Excretion of *Ps. aeruginosa*
1	8/10*
5	5/10
10	2/10
15	2/10
18	2/10
21	0/10

* Numerator: number of excreting mice; denominator: number of infected mice

It should be noted that after the elimination of *E. coli*, anaerobic constituents never appeared in increased numbers, and the occasionally increased *Str. faecalis* counts were never associated with the presence or absence of *E. coli.*

(4) *Antagonism between E. coli and the genera Pseudomonas and Staphylococcus.* The experiments have been extended to Pseudomonas and Staphylococcus, which are frequent accidental organisms of the intestinal flora, and are

supposed to cause pathological conditions associated with the lacking antagonistic activity of the normal flora.

The course of an oral Pseudomonas infection is presented in Table XII.

In the majority of mice the excretion of *Ps. aeruginosa* ceased in a short time. Some mice harboured the organism in low counts for 2—3 weeks. The same result was obtained in animals with an artificial or natural *E. coli* flora.

Implantation of various staphylococcal strains was unsuccessful in untreated or antibiotic-treated mice and chicks. It has been concluded that under the given experimental conditions Pseudomonas and Staphylococcus are unable to colonize in the intestinal tract even in a non-implantation manner as members of the tribe Proteae do.

Discussion

The method used in previous experiments involving the elimination of the intestinal flora by streptomycin treatment and a subsequent establishment of a "monotypic" flora, has been applied for studying the antagonism between *E. coli* and other bacteria.

In spite of the fact that some strains belonging to the tribe Proteae were able to grow in the intestinal tract, the micro-oecological position of these organisms is not doubtful. The lack of implantation or other antagonism, the limited period of excretion and the absence of immunological stimuli to competent cells of the host indicate the accidental character of Proteae. These considerations have been confirmed by the finding that, in contrast to *E. coli*, Proteae can be removed from the intestinal tract with simple washing. This, in agreement with the observations of DUBOS and SCHAEDLER [13], supports our hypothesis [1] as to the close connection between *E. coli* and the intestinal mucosa.

HAENEL [14], who succeeded in isolating Proteus in low counts from each individual of a small group of patients, supposes that Proteus is a constant member of the intestinal flora. In our experience [15] the occurrence of Proteus in humans amounts to about 30 per cent. HAENEL's data may be explained by the fact that at certain periods, presumably due to exogenic, alimentary factors, Proteus shows a higher incidence.

The experimental data for the genus Klebsiella are interesting in connection with the different mechanisms of antagonism. The antagonism between the genera *E. coli* and Klebsiella and that within Klebsiella differ from the "implantation" antagonism existing within the genus *E. coli*. This kind of antagonism is not associated with differences in serotype or characters of the strain, but is merely a matter of preference, which is gained by the first strain to enter the intestinal tract. Implantation antagonism is characterized by an elimination

of the secondary agent 2 to 5 days. The "competition" between *E. coli* and Klebsiella resulted in the predominance of *E. coli*, even in experiments when superinfection was performed with *E. coli*. On the other hand, the elimination of Klebsiella ensued gradually, in 2 to 3 weeks, which is considerably more than the time needed for real implantation antagonism.

An antagonism of the same character occurs when two *E. coli* strains are implanted simultaneously [16].

This kind of antagonism seems analogous to that observed by FRETER [17] between *E. coli* and Shigella, which was explained by a competition for carbohydrates.

If the implantation ability is regarded as a criterion of being a member of the normal intestinal flora, the micro-oecological position of Klebsiella is similar to that of the tribe Proteae. The accidental character of Klebsiella has been confirmed by the finding that its incidence in the intestine is very similar to that of Proteae.

No antagonism has been revealed between micoorganisms regarded as constant constituents and *E. coli*. Indicating the absence of antagonism, increase in *Str. faecalis* or normal anaerobic counts were never noted after the elimination of *E. coli*. DUBOS and SCHAEDLER [18] observed the increase of *E. coli* and *Str. faecalis* in mice in which the original special anaerobic flora had been destroyed. However, no such phenomenon occurred in normal mice. The assumption that the proportion of organisms living together is regulated by an antagonistic mechanism, is evidently erroneous.

The present observations, although far from complete, indicate that certain strains of *Str. faecalis* exert a mutual antagonism similar to that revealed for *E. coli*. These findings are confirmed by JACOBSEN's [19] result, who found that no colonization of *Str. faecalis* could be attained by feeding this organism to normal animals. If similar observations would be available for the normal anaerobic constituents, in other words, if an implantation relationship would be demonstrable between all normal constituents of the flora and the host, we should be able to explain adequately the stability and constant character of these bacteria.

Studies on the antagonistic effect of *E. coli* were extended to other microorganisms encountered in the intestinal flora, which are supposed to multiply and cause pathological conditions when the antagonistic function of the normal flora decreases (during antibiotic therapy).

In these experiments Pseudomonas and Staphylococcus were unable to exert an implantation-like multiplication similar to that observed with Proteae. From these findings it has been concluded that under our experimental conditions these organisms were incapable of colonizing in the unharmed intestinal tract of mice. This observation confirms the opinion that the damaged normal flora and its impaired antagonistic function are by themselves not

responsible for the increase of Pseudomonas and Staphylococcus counts and for the pathogenic role of these bacteria.

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BREAKDOWN OF AMINO ACIDS BY ENTEROBACTERIACEAE

III. CHARACTERISTIC COLOUR REACTIONS IN THE PROTEUS-PROVIDENCIA GROUP

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Summary. It has been shown that in a chemically defined, ferric ammonium citrate-containing medium, bacteria belonging to the Proteus-Providencia group decompose L-tyrosine, L-tryptophan, L-histidine and L-lysine. The breakdown of amino acids is indicated by group-specific colour reactions, which can be used in macro- or micro-tests for identification purposes. Owing to the simple technical procedure, the reactions are suitable for routine work.

In a previous paper it has been shown that various enteric bacteria decompose certain amino acids and form alkaline products [35]. Under certain conditions compounds giving colour reactions with iron salts are produced [36]. The present paper describes investigations into the identification of the Proteus-Providencia group by the use of these colour reactions.

Materials and methods

Bacterial strains, their maintenance and preparation of subcultures have been described previously [34—36].

Ingredients of the medium: NH_4Cl, 1 g; distilled water, 500 ml; KH_2PO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; L-amino acid, 2 g; nicotinamide, 0.01 g; distilled water, 500 ml; 1 per cent $CaCl_2$, 0.1 ml; 1 per cent ferric ammonium citrate, 0.25 ml (to 1 litre histidine solution 0.5 ml, to 1 litre tyrosine or lysine solution 1 ml, 1 per cent ferric ammonium citrate was added after sterilization). After adjusting the pH to 7.5—7.7, the media were sterilized for 15 minutes at 115 °C. When distributed aseptically into tubes, the solutions could be stored at room temperature for 2 months. The following amino acids were examined: L-alpha-alanine, L-cysteine, DL-norvaline, L-leucine, DL-norleucine, DL-isoleucine, L-phenylalanine, L-tyrosine, L-tryptophan, L-histidine, L-aspartic acid, L-glutamic acid, L-arginine, DL-ornithine, L-lysine and DL-oxyproline (DL-amino acids were used in double-strength solutions).

Some amino acids as tyrosine, tryptophan, aspartic acid and arginine dissolved poorly at room temperature. These substances were dissolved in the medium containing the other ingredients by steaming in the Arnold apparatus for ½ hour. On storing at room temperature a precipitate was formed in tyrosine solutions. Therefore after some days this solution was filtered and treated with activated charcoal. Amino acids containing impurities gave yellowish solutions, which, however, were easily decolorized by shaking with activated charcoal and filtering.

In preparing solutions for the microreaction, the medium was adjusted to pH 7.5—7.7, before the addition of the iron salt, then the solutions were steamed in the Arnold apparatus for ½ hour. After cooling, the pH was checked, then to 100 ml tryptophan 0.5 ml, to 100 ml histidine 1 ml, 1 per cent ferric ammonium citrate was added. Finally each 100 ml solution was preserved with 1 ml 1 per cent merthiolate.

On the day of the examination the required volumes were distributed under aseptic conditions into clean 16×160 mm tubes free from residual acid or alkali. For the macrotest generally 2 ml aliquots were used and the tubes were incubated for 4 days at 37 °C. The rapid test was carried out in 0.5 ml aliquots; incubation was performed in a 39°—40 °C water bath.

Changes in the colour of the media were read at hourly and daily intervals in the case of micro- and macro-tests, respectively. For special purposes 5 ml portions of the medium and 6 day incubation periods may be used. The media were inoculated with ample amounts of bacteria from one day agar slant (Russel or desoxycholate citrate agar) cultures with a loop 3 mm in diameter.

Negatively reacting suspensions remained colourless during incubation thus the colour developed by bacteria of the Proteus-Providencia group was easily recognized. The following colour reactions were characteristic of positive reactions; tyrosine, yellow or brown; trytophan, yellow, orange, then brown; histidine, pink, orange or yellow; lysine, greenish. The intensitiy of the colour reaction varied; at reading only negative (colourless) or positive (coloured) results were distinguished.

The proportion of positive reactions increased when *N* NaOH was added to tyrosine or histidine (1 : 10) after incubation and the alkalized suspension was shaken. With tryptophan the characteristic alterations appeared when *N* HCl was added to the suspension (1 : 10).

Results

1. *Colour reaction with ferric ammonium citrate of compounds formed in amino acid metabolism*

Of the listed amino acids colour reactions were obtained with tyrosine, tryptophan, histidine and lysine.

Colour reaction in tyrosine solution. No colour reaction was brought about in 2 ml medium at 37°C for 4 days by 15 strains of each of the following groups or types: *E. coli*, Klebsiella, Citrobacter, Cloaca, Serratia, *S. paratyphi B*, *S. typhi murium*, *S. typhi*, Arizona, *Sh. flexneri* 2, *Sh. boydii* and *Sh. sonnei*. No change was observed after alkalization of the suspensions. Under the same conditions Proteus-Providencia bacteria caused yellow or brown colour reaction in 46 per cent. Immediately after alkalization a vivid green colour appeared, which (after shaking) usually turned into yellow or brown in 1 minute. At the same time a flaky precipitate was formed, which sedimented in 15 to 30 minutes and left over a brownish or cherry-red deposit under the translucent, yellow or brown supernatant. Most positive results (94 per cent) were obtained when readings were based on the colour of the supernatant one day after alkalization (Table I).

Colour reaction in tryptophan solution. Uniformly negative results (no colour in 5 ml L-tryptophan solution at 37°C within 5 days) were given by 444 enteric bacterial strains (137 *E. coli*, 28 Klebsiella, 7 Aerobacter, 15 Citrobacter, 15 Cloaca, 12 Serratia, 7 *S. paratyphi A*, 15 *S. paratyphi B*, 19 *S. thypi murium*, 21 *S. typhi*, 26 other Salmonella, 15 Arizona, 9 *Sh. dysenteriae*, 74 *Sh. flexneri*, 15 *Sh. boydii*, 14 *Sh. sonnei*, 7 Hafnia and 9 Pseudomonas cultures). No precipitate was formed after acidifying the media, and, except for indole positives, the cultures remained colourless. Addition of acid caused a pinkish cherry-red colour without precipitation in 89 *E. coli*, 7 Aerobacter, 4 *Sh. dysenteriae* and 4 *Sh. flexneri* 2 cultures. This colour reaction has been regarded as a positive indole test. However, with the conventional indole reaction [18] considerably more positive results were obtained (*e.g.* 130 out of 137 *E. coli*

Table I

Breakdown of L-tyrosine by Proteus-Providencia bacteria

2 ml 0.2 per cent L-tyrosine, pH 7.7, loopful inoculum

	No. of strains	Percentage of positive cultures after							
		1	2	3	4	4 days' incubation and alkalization			
		days incubation				green colour	after shaking		
							immediately	after 20 min.	next day
P. vulgaris	37	—	5	22	57	92	92	92	92
P. mirabilis	38	—	3	3	26	63	73	84	94
Morganella	41	—	—	10	24	97	95	95	97
Rettgerella	33	—	3	21	51	82	79	82	88
Providencia	34	—	—	24	76	100	97	100	100
Total	183	—	2	15	46	86	87	90	94

strains). Thus in our medium only part of the indole positive strains produced this substance.

In the course of the 3-day incubation period, Proteus-Providencia strains gave yellow, orange, then brown colour reaction in 98 per cent (Table II). After the addition of acid, a brown or cherry-red granular precipitate was formed in tryptophan-decomposing suspensions. After a rapid sedimentation of the precipitate, the translucent supernatant was yellow, brown, orange or cherry-red in colour.

None of the 18 *P. vulgaris*, 15 *P. mirabilis*, 17 Morganella, 17 Rettgerella, 12 Providencia, 20 *E. coli*, 10 Klebsiella, 10 Citrobacter, 10 Cloaca, 10 Serratia,

Table II

Breakdown of L-tryptophan by Proteus-Providencia bacteria

5 ml 0.2 per cent L-tryptophan, pH 7.5, loopful inoculum

	No. of strains	Percentage of positive cultures after		
		1	2	3
		days' incubation		
P. vulgaris	37	2	94	100
P. mirabilis	38	13	66	97
Morganella	41	12	73	97
Rettgerella	33	—	57	97
Providencia	33	3	94	100
Total	182	7	75	98

5 *S. paratyphi B*, 5 *S. typhi murium*, 5 *S. typhi*, 5 Arizona, 5 Hafnia and 5 Pseudomonas cultures induced a colour reaction during the 5-day incubation period in 5 ml D-tryptophan solution. After the addition of acid only a few cultures (3 Morganella and 4 Providencia) showed a faint pinkish colour, presumably due to a very weak indole production. Thus the tryptophanase enzyme responsible for the breakdown of tryptophan may be considered specific.

The appearance of the colour reaction was examined in solutions adjusted to pH 5.1—7.5. The results are summarized in Table III. It has been shown that most strains exert tryptophanase activity at a relatively low pH value. Analogous findings have already been described [13, 33, 41].

Strains of various activity can easily be distinguished when the reaction is performed in 5 ml aliquots of media (Table II). For routine purposes the use of smaller amounts is more advantageous. In 2 ml L-tryptophan the colour reaction of Proteus-Providencia strains appeared after one day in 96 per cent, after 2 days in 98 per cent.

Table III

Tryptophan deamination by Proteus-Providencia bacteria at various pH values

	No. of strains	No. of positive cultures at pH				
		5.1	5.7	6.2	6.7	7.5
P. vulgaris	15	7	15	15	15	15
P. mirabilis	15	8	15	15	15	15
Morganella	15	14	15	15	15	15
Rettgerella	15	14	14	15	15	15
Providencia	15	12	14	15	15	15

It is known that some carbohydrates, especially glucose, inhibit the biosynthesis of certain adaptive enzymes. The breakdown of amino acids greatly decreases or ceases when the bacteria are harvested from glucose-containing media [5, 15, 16, 30]. It has therefore been examined whether cultures grown on carbohydrate-containing media are suitable for the reaction. The results were similar to the control tryptophan test when the tubes were inoculated from Russel or desoxycholate citrate agar, or when glucose and other carbohydrate-containing tryptophan solutions were used. The interfering action of L-serine [27] was also examined in a solution containing 0.2 per cent L-serine and 0.2 per cent L-tryptophan. Under the given experimental conditions L-serine exerted no inhibiting effect on the appearance of the colour reaction.

Colour reaction in histidine solution. Uniformly negative results were obtained with 321 enteric bacterial strains (75 *E. coli*, 28 Klebsiella, 7 Aero-

bacter, 15 Citrobacter, 15 Cloaca, 12 Serratia, 7 *S. paratyphi A*, 15 *S. paratyphi B*, 10 *S. typhi murium*, 4 *S. kunzendorf*, 3 *S. bareilly*, 3 *S. anatum*, 15 Arizona, 5 Hafnia, 8 *Sh. dysenteriae*, *Sh. flexneri* type 1, 2, 3, 4, 10 strains of each, 8 *Sh. flexneri* 5, 6 *Sh. flexneri* 6, 10 *Sh. boydii*, 9 *Sh. sonnei* and 8 Pseudomonas cultures). These strains caused no change in 5 ml amounts of L-histidine medium incubated at 37°C for days. After the addition of alkali only a white, flaky precipitate was formed. Under similar conditions 61 per cent of Proteus-Providencia bacteria caused a pink, orange or yellowish colour reaction. After alkalization, in each positive tube a cherry-red deposit was formed and the supernatant, which became fainter immediately after alkalization, took on a yellowish-golden colour. Considering the changes that occurred after alkalization, Proteus-Providencia bacteria were positive in 84 per cent (Table IV).

Table IV

Breakdown of L-histidine by Proteus-Providencia bacteria

5 ml 0.2 per cent L-histidine, pH 7.5, loopful inoculum; after 6 days incubation, 0.5 ml *N* NaOH was added then the culture was reincubated at room temperature overnight

	No. of strains	Percentage of positive cultures after days of incubation						
		1	2	3	4	5	6	7
P. vulgaris	32	—	38	53	75	81	90	100
P. mirabilis	38	—	26	50	60	74	76	84
Morganella	41	14	31	36	49	61	61	75
Rettgerella	33	9	12	18	18	18	36	88
Providencia	32	—	6	16	25	44	44	75
Total	176	5	23	35	46	56	62	84

The reaction in D-histidine solution of 8 *P. vulgaris*, 6 *P. mirabilis*, 6 Morganella, 3 Rettgerella and 2 Providencia strains, which were able to decompose L-histidine, was also examined. A definitely positive reaction was never observed. Minimal colour changes occurred in 1 *P. mirabilis* culture after 4 days incubation, in 2 *P. vulgaris* and 3 *P. mirabilis* cultures the deposit turned faint cherry-red one day after alkalization.

The sensitivity to acid of the histidase enzyme responsible for L-histidine breakdown, varied more definitely according to subgroups than the sensitivity to acid of tryptophanase did (Table V).

Colour reaction in lysine solution. The 180 enteric bacterial strains examined for tyrosine breakdown brought about no colour reaction in 2 ml L-lysine during 4 days incubation. The suspensions remained unaltered after the addition of acid or alkali. The members of the Proteus-Providencia group sometimes

Table V

Histidine deamination by Proteus-Providencia bacteria at various pH values

	No. of strains	No. of positive cultures at pH				
		5.1	5.7	6.2	6.7	7.5
P. vulgaris	20	12	20	20	20	20
P. mirabilis	20	19	20	20	20	20
Morganella	20	3	17	20	20	20
Rettgerella	20	20	20	20	20	20
Providencia	20	12	13	14	14	14

Table VI

Breakdown of L-lysine by Proteus-Providencia bacteria

2 ml 0.2 per cent L-lysine, pH 7.7, loopful inoculum

	No. of strains	Percentage of positive cultures after days of incubation			
		1	2	3	4
P. vulgaris	38	—	—	2	2
P. mirabilis	37	—	—	—	16
Morganella	41	—	5	5	5
Rettgerella	33	54	64	67	67
Providencia	34	38	41	41	41
Total	183	17	20	21	24

caused a greenish reaction, which was decolorized by alkali but left unaltered by acid treatment (Table VI).

Comparison of results in various amino acid solutions; colour reaction in mixed amino acid solutions. Of the examined Proteus-Providencia strains, a colour reaction was given by 94 per cent in L-tyrosine, 98 per cent in L-tryptophan, 84 per cent in L-histidine and 24 per cent in L-lysine solutions. Thus the best results were obtained with L-tryptophan. The tryptophan negative 1 *P. mirabilis*, 1 Morganella and 1 Rettgerella strains were unable to attack other amino acids. Thus no uniform positivity was obtained in this group of enteric bacteria, even when the cultures were examined simultaneously in 4 different amino acid solutions.

As mentioned above, the presence of serine did not inhibit L-tryptophan decomposition. It has, therefore, been assumed that instead of an interference among the amino acids, the number of positive results might be increased by the use of a mixture containing L-tyrosine, L-tryptophan, L-histidine and L-lysine. For the experiments equal amounts of the above amino acid solution

were mixed and 2 ml aliquots were inoculated with 182 Proteus-Providencia and 125 other enteric bacterial strains. The latter cultures representing members of the earlier listed groups, caused no colour reaction during 2 days incubation. Of Proteus-Providencia bacteria 176 (97 per cent) caused a yellow or brown colour to appear. The tryptophan-negative cultures were negative also in the mixed solutions.

Accordingly, Proteus-Providencia cultures can be recognized in the highest proportion when the reaction is carried out in 2 ml tryptophan solution with an incubation period of one day.

Colour reactions in solid media. Members of the Proteus-Providencia group may cause colour reactions in solid media containing amino acids. For these experiments the following amino acids were dissolved in conventional peptone-meat infusion agar (pH 7.2 to 7.4) so as to give final concentrations of 0.2 per cent (L-amino acid) or 0.4 per cent (racem form): glycine, L-alpha-alanine, L-serine, L-cysteine, L-cystine, DL-methionine, L-threonine, DL-valine, DL-norvaline, DL-norleucine, L-leucine, DL-isoleucine, L-phenylalanine, L-tyrosine, DL-tryptophan, DL-histidine, L-oxyproline, DL-aspartic acid, L-asparagine, L-glutamic acid, L-glutamine, L-arginine, L-lysine. Then to 100 ml medium 0.03 ml of 1 per cent ferric ammonium citrate was added. The medium was distributed into test tubes in 5 ml portions and left to solidify in a slanted position. Needle point amounts of *P. vulgaris*, Providencia and *E. coli* cultures (5 strains each) were streaked onto the slants. The media were observed daily during the 3 day incubation period.

E. coli cultures caused no colour reaction. In media containing cysteine, tyrosine, tryptophan or histidine all the examined *P. vulgaris* and Providencia strains gave rise to a yellowish, then brownish colour diffusing into the medium and staining the culture itself. In cystine and lysine media only the upper third of the slant was coloured. Of the other amino acids, threonine, valine, norleucine and oxyproline gave colour reactions with some *P. vulgaris* cultures. This type of colour appeared in the butt of the medium.

2. Rapid tests

The presence of free oxygen enhances the oxidative deamination of amino acids. Therefore, the rate of reaction increases when under identical conditions smaller volumes of amino acid solution are used. In 0.5 ml volumes in 16×160 mm test tubes the colour indicating of amino acid breakdown appears in some hours. On the basis of this experience very simple methods can be made use of for the rapid test. For microtests, L-tryptophan and L-histidine have been found satisfactory.

Microtest for assaying L-tryptophan breakdown. Except for the Proteus-Providencia group, all the Enterobacteriaceae strains tested (20 *E. coli*, 20

Klebsiella, 15 Citrobacter, 15 Cloaca, 12 Serratia, 15 *S. paratyphi B*, 15 *S. typhi murium*, 15 *S. typhi*, 15 Arizona, 30 *Sh. flexneri*, 13 *Sh. boydii*, 15 *Sh. sonnei* cultures) gave negative results during the one-day observation period, in that the colourless or whitish suspension remained unchanged. Proteus-Providencia cultures gave positive reactions within 6 hours in 98 per cent. At first a faint yellow colour appeared, then its intensity and the number of positive

Table VII

Rapid tryptophan test

0.5 ml 0.2 per cent L-tryptophan, pH 7.5, loopful inoculum

	No. of strains	Percentage of positive cultures after hours of incubation					
		1	2	3	4	5	6
P. vulgaris	37	8	100	100	100	100	100
P. mirabilis	38	5	92	97	97	97	97
Morganella	40	—	5	45	80	97	97
Rettgerella	33	3	48	84	91	94	97
Providencia	34	3	47	100	100	100	100
Total	182	4	58	84	94	97	98

tubes increased gradually. After some hours considerable number of suspensions took a deep brown colour (Table VII). To colourless suspensions 0.1 ml *N* HCl was added. After 5 to 10 minutes in 9 out of 20 *E. coli*, in 4 out of 15 *Sh. flexneri* 3, and in 6 out of 13 *Sh. boydii* cultures a paler or deeper pink colour appeared. This reaction was regarded as a rapid indole test. The addition of acid,

Table VIII

Rapid histidine test

0.5 ml 0.2 per cent L-histidine, pH 7.5, loopful inoculum

	No. of strains	Percentage of positive cultures after					
		1	2	3	4	5	alkalization
		hours' incubation					
P. vulgaris	37	65	84	94	97	97	97
P. mirabilis	38	60	84	89	94	97	97
Morganella	40	5	12	32	42	42	57
Rettgerella	33	69	78	85	91	94	100
Providencia	34	47	71	91	91	91	100
Total	182	48	64	72	77	79	86

however, did not increase the number of positive reactions among Proteus-Providencia bacteria.

Microtest for assaying histidine breakdown. Bacteria used for the tryptophan microtest were examined. The 200 Enterobacteriaceae strains gave uniformly negative results during the one day incubation period. After 6 hours incubation, 0.1 ml portions of *N* NaOH were mixed with the suspensions. This caused a flaky precipitation; the suspensions remained colourless or whitish. After sedimentation overnight, whitish, greyish or yellowish deposits and a colourless supernatant could be observed. Almost half of the Proteus-Providencia cultures gave a pale pink reaction after one hour. On further incubation the intensity and number of colour reactions increased (Table VIII). The proportion of positive results increased when 0.1 ml *N* NaOH was added to each tube and after vigorous shaking the tubes were returned into the water bath. Addition of NaOH may turn the colourless Proteus-Providencia culture yellow and/or may cause the formation of a slight amount of rusty or cherry-red deposit.

Table IX

Rapid test in tryptophan—histidine mixture

0.25 ml 0.2 per cent L-tryptophan+0.25 ml 0.2 per cent L-histidine, pH 7.5, loopful inoculum

	No. of strains	Percentage of positive cultures after hours of incubation		
		1	2	3
P. vulgaris	37	65	97	97
P. mirabilis	38	71	97	97
Morganella	41	14	56	90
Rettgerella	33	15	67	91
Providencia	35	17	91	100
Total	184	37	81	95

Microtest in the mixture of tryptophan and histidine solutions. Equal volumes of 0.2 per cent L-tryptophan and 0.2 per cent L-histidine were mixed and the solution was distributed in 0.5 ml amounts in 16×160 mm tubes. Then in each tube a loopful of culture was suspended. The tubes were incubated for 3 hours in a 39—40°C water bath. Readings were made at one hour intervals. After the addition of 0.1 ml *N* NaOH, the tubes were left to stand at room temperature. Readings were performed after 15 minutes and on the next day. The examined 200 enteric bacterial strains gave uniformly negative reactions. Proteus-Providencia cultures showed a yellow, pink or orange

colour after 1 hour in 37 per cent. On further incubation the number of positive cultures and the intensity of the colour reaction increased. As a whole, after 3 hours incubation 95 per cent of the 182 Proteus-Providencia strains gave a positive reaction (Table IX). After alkalization the intensity of the reaction indicating the breakdown of amino acids may increase and further strains may turn out to be positive.

On the basis of the present results it may be concluded that for everyday routine purposes the detection of tryptophanase activity in 2 ml solution with incubation overnight, seems most suitable. The procedure is simple as it requires only routine bacteriological manipulations (seeding, incubation in the thermostate and reading of colour reaction on the next day). When a rapid identification of Proteus-Providencia bacteria is necessary, the microtest carried out with strains from selective media in the mixture of L-tryptophan and L-histidine is recommended. The microtest is also a simple procedure, only the use of a water bath and readings at one hour intervals are somewhat laborius.

Discussion

It has been known for nearly 60 years that putrifying bacteria are able to decompose amino acids by deamination or decarboxylation, and may produce acetic acid from glycine, or succinic and propionic acids from aspartic acid [28]. At the same time it has been shown that amino acids tend to fall in a definite order according to the ability of Proteus bacteria to attack them. Beginning with the member most easily decomposable, the order of these substances is: aspartic acid, leucine, amino-valerianic acid, phenylalanine, tyrosine, arginine, glycine, alanine; as decomposition products, organic acids, or aldehydes and ammonia are formed [29]. In the decades following the time of the first investigations, a number of authors have dealt with the amino acid-decomposing activity of these bacteria [1, 4, 22, 24, 32, 42, 43]. It has been shown that *P. vulgaris* oxidizes practically all natural amino acids [4, 42] by enzymes catalyzing the oxidative deamination of these substances into the corresponding keto-acids and ammonia [42, 44, 45].

The characteristic enzyme activity of the Proteus group allows its use for identification purposes. From phenylalanine these bacteria rapidly produce large amounts of phenylpyruvic acid, which is simple to detect on the basis of its colour reaction with ferric ammonium sulphate. HENRIKSEN's phenylalanine deaminase test [21] has proved very suitable for the differentiation of Proteus from other enteric bacteria [3, 7—10, 17, 23, 31, 37, 38, 40). According to SINGER and VOLCANI [39] the members of the Proteus-Providencia group deaminate tryptophan, and the produced indole-3-pyruvic acid gives a persisting colour reaction with ferric chloride. Therefore, for the routine iden-

tification of the Proteus-Providencia group, these authors recommended the tryptophanase test [39]. Modified techniques have been elaborated in order to render shaking unnecessary [26] or to carry out the reaction with paper strips impregnated with amino acid solutions [2, 12, 17].

Examining the breakdown of 23 amino acids by different enteric bacteria, SINGER and VOLCANI found that decomposition products formed as a result of the action of Proteus-Providencia enzymes gave a colour reaction with ferric chloride only in case of DL-methionine, L-leucine, DL-isoleucine, DL-norleucine, DL-norvaline, L-phenylalanine, L-tryptophan and L-histidine. The colour reactions, with the exception of the cherry-red colour given by the decomposition product of tryptophan, were not durable [39]. These findings seemed to indicate that there were no further amino acid deaminase colour reactions suitable for the identification of the Proteus-Providencia group. Our results have shown that with histidine decomposition products a durable colour reaction can be obtained, which, however, rapidly fades when SINGER and VOLCANI's method is used. With our techniques persisting colour reactions were obtained also in lysine and tyrosine solutions, the metabolic products of which give non-characteristic reactions with ferric chloride.

As to the colour reaction of further amino acids, in addition to the difference between our findings and the data in the literature, it was striking that phenylalanine should consequently have yielded negative results. In our opinion the differences were due to differences in the applied techniques. Under suitable conditions, bacteria of the Proteus-Providencia group deaminate all natural amino acids and form keto-acids and ammonia[42, 44, 45]. Enol forms of keto-acids and perhaps endiols give coloured complex compounds with ferric chloride [6, 19, 20]. The formation of complexes depends on various factors, such as the pH of the medium and the iron salt reagent. Thus, on the basis of a negative colour reaction no conclusions can be drawn as to the lack of deamination. In this way not only the variability of results can be explained, but by elaborating modified methods or perhaps by applying other metal salt reagents, a possibility offers itself for finding new tests suitable for the identification of Proteus-Providencia strains.

The colour reactions given by this group of bacteria result from the activity of deaminase enzymes. With the simultaneous production of ammonia, tyrosine is broken down to p-hydroxyphenyl-pyruvic acid [39], tryptophan to indole-3-pyruvic acid [39], histidine to urocanic acid and formamide [30, 37]. As to lysine, only data for a decarboxylation reaction yielding cadaverine and CO_2 are available [16]. It is, however, known that Proteus-Providencia bacteria give a negative lysine-decarboxylase reaction [14]. Thus in our experiments an oxidative deamination must have taken place. This is confirmed by the accelerating effect of air (oxygen) on the reaction rate, the alkaline pH optimum, iron salt reactions characteristic of enol and endiol forms of keto-acids; and

occasional definite and rapid changes in the colour of complex compounds. With the alkali test [35] it can be shown that during the reaction alkaline products are formed, and, further the negative ninhydrin reaction excludes the presence of amines. PROOM and WOIWOD, who have shown that amine production is characteristic of the Proteus group, have been unable to reveal this property in Rettgerella and in most Providencia cultures [31, 31a].

The advantage of the methods presented in this paper over the large number of rapid Proteus-Providencia tests suitable for routine purposes, must finally be discussed. Most rapid tests require an agar culture grown for one day. Thus, for carrying out the reaction, which itself takes only 10 to 60 minutes, in reality a 24 hour period is needed. Our method can be performed with inocula obtained directly from selective plates, and therefore it can be regarded as a really rapid procedure for recognizing Proteus-Providencia strains. In addition, its simple technique presents a further advantage in the routine laboratory. The iron salt is incorporated in the medium, thus there is no need to add the reagent or reagents separately. Finally, in identifying atypical, for example lactose positive [42a], Proteus-Providencia cultures, our method suits itself well for the examination of tyrosine, histidine and lysine breakdown.

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STUDIES ON KLEBSIELLA INFECTIONS BY PHAGE DETECTION AND PHAGE TYPING

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Summary. In order to elucidate the aetiology of severe enteric infections in premature infants, bacteriological examinations have been supplemented with the detection of specific phages. It has been shown that this method suits itself well for revealing the aetiological agent. By adapting phages isolated for diagnostic purposes to Klebsiella strains, a typing scheme for epidemiological investigations has been elaborated. The isolated phages were characterized by phage morphology, heat sensitivity, lytic activity and serological properties.

By means of specific phages Klebsiella strains of various origin were divided into 5 phage groups and 13 types.

The phage type distribution of Klebsiella strains was examined in nosocomial outbreaks of enteritis and in sporadic cases.

Among the examined 802 Klebsiella cultures 546 (68 per cent) typable strains occurred. Strains from hospital outbreaks were phage sensitive in 73, those from sporadic cases in 40, per cent.

The association between K antigens and phage types has been investigated.

Isolation of phages acting on various Klebsiella strains has been reported by several authors. From the faeces of a septicaemic patient CAUBLOT [1] in 1924 isolated for therapeutic purposes a phage that lyzed Friedländer's bacillus. HADLEY [2] isolated a Klebsiella phage from sewage, by which S and R variants could be distinguished. From *B. rhinoscleromatis* strains PRASEK and PRICA [3] obtained specific rhinoscleroma phages capable of giving rise to noncapsulated forms in homologous cultures. The noncapsulated forms were used as antigens for diagnostic agglutination test. Rhinoscleroma phages were isolated by GERKES [4] from 7 week broth and by IZRAITEL [5] from 5—7 week agar cultures.

Differentiation of Klebsiella strains with specific phages was attempted by RAKIETEN *et al.* [6] who found a close association between capsular antigens and sensitivity to phages isolated from sewage. With specific phages Klebsiella types A, B and C were distinguished. Type B phages lyzed also Aerobacter cultures. PARK [7] described in detail a phage of unknown origin, which was specific for Klebsiellae.

ADAMS and PARK [8] studied the nature of the halo surrounding plaques produced on phage action. The experiments showed that the halo was brought about by an enzyme, which diffused more rapidly than the phage and influenced the growth of bacteria. MATSELIUCH [9]obtained by adaptation a phage specific for the used rhinoscleroma strain.

EUSTATZIOU *et al.* [10] investigated the association between serotype and lysogenic properties of 68 Klebsiella strains. Among the strains 8 per cent lysogenic cultures occurred, which yielded phages acting exclusively on bacteria belonging to the corresponding serotype. In the opinion of these authors the enzyme encountered in the phage—cell system causes difficulties in phage typing. GAVRILOVITCH [11] examined with phages isolated from lysogenic rhinoscleroma cultures the association between phage sensitivity and lysogenicity of 7 rhinoscleroma cultures. On the basis of these properties, he divided the strains into 4 groups.

Data in the literature indicate that, although differentiation of Klebsiellae by means of phages has been attempted, at present there is no method suitable for epidemiological purposes. The present work was undertaken to establish a practical division of the Klebsiella group by the isolation of specific phages.

I. DETECTION OF PHAGES

In a department for premature infants the number of patients dying from enteritis highly increased in 1961—62. At the same time the incidence of peritonitis and of secondary intestinal perforations also increased. Simultaneously with the highest incidence of peritonitis cases of enteritis with unusual course occurred. At autopsy of 10 infants fibrinopurulent peritonitis was revealed. Neither routine bacteriological nor virological examinations elucidated the aetiology. Subsequently, in addition to bacteriological examinations, the detection of phages was attempted. The methods and results are presented as follows.

Materials and methods

The examinations were performed between November 20 and December 7, 1961, and between February 12 and April 4, 1962. Altogether 1131 faecal specimens originating from 369 infants were examined.

Bacteriological examinations. Enteric pathogens were isolated on eosin-methylene blue or Endo and on desoxycholate citrate agar. The isolated cultures were identified by biochemical reactions. With some strains mouse inoculation experiments were also carried out. Antibiotic sensitivity was tested with "Biotest" paper discs (Institute for Serobacterial Production and Research, Human, Budapest). In addition, the sensitivity reactions to other, recently introduced antibiotics were examined.

Phage examinations. Isolation of phages and determination of phage sensitivity of bacterial strains were performed from 174 faecal samples obtained from 79 premature infants. Methods for phage isolation and detection were as described in a previous paper for *E. coli* strains [12]. The indicator strains were partly "standard" laboratory strains of known pathogens, partly strains isolated from faecal samples during the epidemics.

Phage sensitivity of bacteria was tested with phages isolated during the epidemic and with 38 different phages active against various enteric bacteria. The latter set of phages was isolated in previous years from the faeces of infants suffering from enteritis.

Results

Identification of the causative agent. Lactose-positive mucoid colonies growing from most faecal samples were sensitive to standard phages specific for Klebsiella strains. In phage isolation experiments, 20 of the faecal samples (11 per cent) yielded specific phages acting on Klebsiella strains. Cultures isolated on the basis of phage sensitivity and phage detection were later identified by means of biochemical reactions and animal inoculation.

Klebsiella strains were isolated from 348 out of 1131 samples (31 per cent) originating from 198 out of 369 infants (54 per cent). The highest number of infections occurred at the conditioned department, where Klebsiella was recovered from 188 out of 447 samples (42 per cent), originating from 101 out of 147 infants (69 per cent).

Comparison of laboratory findings with clinical data. Of 198 premature infants positive for Klebsiella 48 (24 per cent) had severe or moderate enteritis. Mild infections occurred in 51 infants (26 per cent). Ten infants (5 per cent) died; 89 infants (45 per cent) were symptomless carriers.

Table I presents the distribution of 218 phage sensitive and phage resistant Klebsiella strains isolated from the faeces of infants with enteritis and from symptomless carriers. In the former group of infants the incidence of phage sensitive strains was significantly higher than in the carriers. Six cases were examined bacteriologically post mortem; 5 of them yielded Klebsiella from various organs; in the remaining 1 case culturing was attempted only from the spleen.

Antibiotic sensitivity. Of 149 strains 7.3 per cent was resistant and 24.1 per cent moderately sensitive to neomycin; 2.6 per cent was resistant and 61.7 per cent moderately sensitive to polymyxin B. According to UJVÁRY [13] in 1959 only 0.3 per cent of the strains was resistant to neomycin. The 1962 data of LOSONCZY *et al.* [14] are almost identical with our result, their nosocomial strains were neomycin resistant in 7.8 per cent. All strains were resistant to the other examined antibiotics.

Table I

Distribution of Klebsiella strains according to phage sensitivity

Origin of strains	Phage sensitive strains		Phage resistant strains		Total	
	No.	per cent	No.	per cent	No.	per cent
Patients	93	61	23	35	116	53
Symptomless excreter	60	39	42	65	102	47
Total	153	100	65	100	218	100

$\chi^2 = 10.82$; P = 0.1 per cent

Isolation of phages. Ten of the isolated 20 specific Klebsiella phages were recovered from patients not shown to excrete Klebsiellae at the time of phage isolation. At subsequent examinations, however, Klebsiella strains were isolated also from these patients. From symptomless carriers 10 additional specific phages were recovered. No association was found between the severity of infection and the presence of virulent phages.

From the phage sensitivity of the cultured bacteria and the presence of virulent phages, conclusions can be drawn as to the responsibility for the infection of the recovered organism. Bacteria isolated on the basis of phage sensitivity may subsequently be identified by bacteriological methods. The pathogenic role of Klebsiella in the studied epidemic was confirmed by the following findings.

(1) Klebsiella strains or Klebsiella phages were isolated from nearly all faecal samples.

(2) With one exception, post mortem examinations of various organs yielded Klebsiella in pure culture.

(3) Other pathogenic organisms were found neither in faecal samples nor in the organs.

(4) Other, mainly *E. coli*, strains isolated from faecal samples were heterogeneous as to phage pattern.

(5) Antibiotic therapy aimed against Klebsiella strains was successful in most cases.

Detection of specific phages is important for establishing the proper aetiological diagnosis. Phages may be responsible for, and, alternatively, may be consequences of, the change of a facultatively pathogenic organism into a pathogenic agent. Induction of toxin production [15—18] and increased virulence of *E. coli* [12, 19] have been attributed to phage action.

II. PHAGE TYPE DETERMINATION

Materials and methods

Phages used for typing were isolated from faecal samples during outbreaks of enteritis occurring between October, 1961, and June, 1963, in the premature and infants' departments of two hospitals. The isolated phages were examined for plaque morphology, heat sensitivity, lytic activity and serological properties.

Size and morphology of phages were determined by GRATIA's agar layer method [20]. The phages were diluted so as to give distinct plaques. One plaque of each phage was subcultured in order to determine the stability of plaque types. Although on subcultivation the plaques retained their original type, mutations or variations were often observed. The diameter and morphology of plaques were read after an incubation at 37°C for 18 hours.

In heat sensitivity testing the phages were exposed in Hartley broth to different temperatures ranging from 60 to 80°C for 30 minutes.

In order to show serological relationships, immune sera were prepared against all phages. Neutralization tests were carried out with all sera and phages. In the case of positive reactions the K values were determined by the method of BURNET *et al.* [21].

The 12 Klebsiella and 1 Enterobacter phages were selected on the basis of lytic activity and serological properties. The propagated phages were used either undiluted or in the critical test concentration (the highest dilution which produced confluent lysis of the homologous strain).

The examined strains were isolated partly from the faeces of infants with enteritis treated in the above hospitals, partly from clinical samples of urine, CSF, materials from eye and skin diseases, sputum and pus, sent to the Department of Bacteriology of this Institute. In addition, the phage sensitivity of standard Klebsiella strains representing K antigens 1 to 30, was tested.

Altogether 803 Klebsiella and 99 Enterobacter strains were examined.

Results and discussion

Examinations of plaque morphology revealed 7 plaque types differing in size and morphology. Part of the phages produced a faint halo surrounding the clear plaque. Some plaque types are presented in Figs. 1—5.

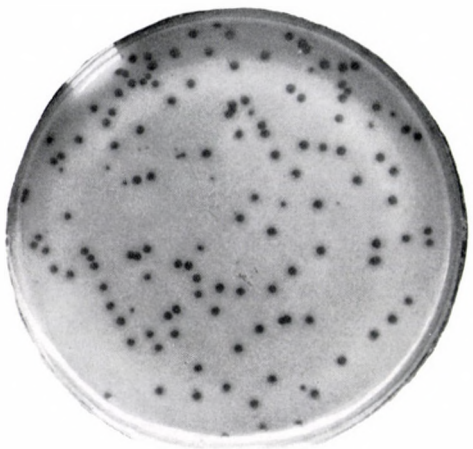

Fig. 1. Plaques 3 mm in diameter, showing clear areas with entire edge surrounded by a definite halo (Klebsiella phage 328)

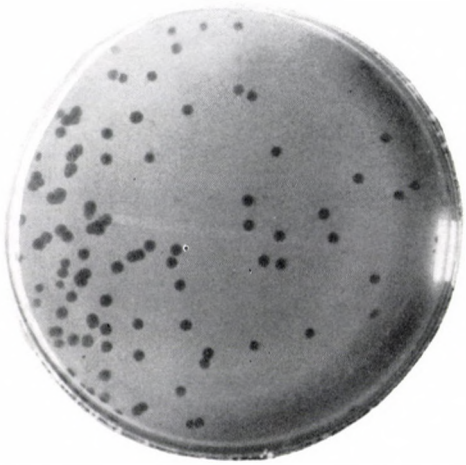

Fig. 2. Plaques 4 mm in diameter showing clear areas with entire edge (Klebsiella phage 4)

By heat sensitivity testing 2 groups were distinguished: 9 phages were inactivated at 70°C (Klebsiella phages 2, 6, 42, 328, 181, 937, 832, 765 and Enterobacter phage 14); the remaining 4 Klebsiella phages (4, 106, 165, 171) were destroyed at 80°C.

Serological determinations showed that the K values of antiphage sera against the homologous phages varied between 32 and 200. On the basis of the cross-neutralization reactions and K values, Klebsiella typing phages were divided into 5 serological groups (A, B, C_1, C_2, D) (Table II). A minor relationship was revealed between phages belonging to groups C_1 and C_2. The serum

prepared against phage 14 acting on Enterobacter strains, did not react with any of the Klebsiella phages, therefore this phage was regarded as belonging to a separate serogroup.

Phage sensitivity testing of 802 Klebsiella and 99 Enterobacter strains showed that the 12 Klebsiella phages lyzed only Klebsiella strains, while the phage adapted to an Enterobacter strain acted on Enterobacter but not on Klebsiella cultures.

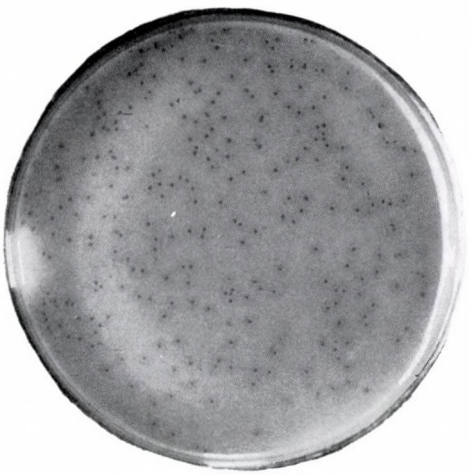

Fig. 3. Small plaques surrounded by a large halo (Klebsiella phage 171)

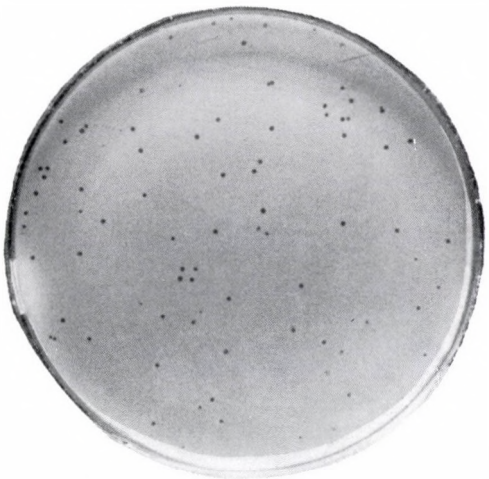

Fig. 4. Plaques 2 mm in diameter showing clear areas with entire edge (Klebsiella phage 42)

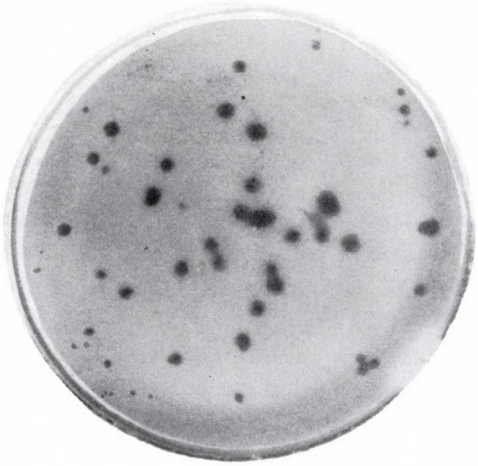

Fig. 5. Irregularly shaped and sized plaques (Enterobacter phage 14)

Table II

Serological classification of Klebsiella phages

Phage	Serological groups	4	328	2	42	6	171	106	165	181	832	937	765
		Constant for the rate of antiphage serum neutralization (K value)											
4	A	41	24	—	—	—	—	—	—	—	—	—	—
328		22	200	—	—	—	—	—	—	—	—	—	—
2	B	—	—	68	55	—	—	—	—	—	—	—	—
42		—	—	16	133	—	—	—	—	—	—	—	—
6	C_1	—	—	—	—	50	33	—	—	—	<1	—	—
171		—	—	—	—	26	74	9	5	<1	<1	—	—
106	C_2	—	—	—	—	2	>1	32	31	55	—	—	—
165		—	—	—	—	8	<1	20	66	29	—	—	—
181		—	—	—	—	<1	>1	28	52	59	—	—	—
832	D	—	—	—	—	<1	<1	—	—	—	70	22	78
937		—	—	—	—	<1	<1	—	—	—	68	136	33
765		—	—	—	—	<1	<1	—	—	—	34	32	172

Of the 99 Enterobacter strains 40 (40.4 per cent) were lyzed by the phage adapted to *Enterobacter cloacae* strain 14. Although Klebsiella and Enterobacter strains can be distinguished on the basis of motility and biochemical behaviour, in certain cases their classification may be performed more rapidly by the use of specific phages.

Strains belonging to the Klebsiella group were divided into 5 phage groups and 13 phage types. The phage typing scheme showing the reactions obtained with 12 typing phages included in serological groups A, B, C_1, C_2 or D is presented in Table III.

The distribution of phage types within the 5 phage groups is as follows.

Group I includes 5 types (1a, 1b, 1c, 1d, 1e)

Group II includes 2 types (2a, 2b)

Group III includes 2 types (3a, 3b)

Group IV includes 3 types (4a, 4b, 4c)

Group V includes 1 type (5a).

There was a close association between the phage sensitivity of strains and the serological group of phages.

Table IV shows the phage group and phage type distribution of 802 Klebsiella strains. The majority of strains (598) originated from faecal samples; these strains could be typed in 72 per cent. Nose and throat samples yielded 82 strains, which were typable also in 72 per cent. A similar proportion (71 per cent) of typable strains occurred among cultures isolated from various objects in the wards. Strains originating from other clinical materials and the standard

Table III

Phage typing scheme for the Klebsiella group

Phage		Serological groups											
		A		B		C_1		C_2			D		
		Typing phages											
group	type	4	328	2	42	6	171	106	165	181	937	832	765
I	1a	+	—	—	—	—	—	—	—	—	—	—	—
	1b	+	+	—	—	—	—	—	—	—	—	—	—
	1c	—	+	—	—	—	—	—	—	—	—	—	—
	1d	+	—	+	—	—	—	—	—	—	—	—	—
	1e	+	+	+	—	—	—	—	—	—	—	—	—
II	2a	—	—	+	—	—	—	—	—	—	—	—	—
	2b	—	—	+	+	—	—	—	—	—	—	—	—
III	3a	—	—	—	—	+	—	—	—	—	—	—	—
	3b	—	—	(+)	—	+	+	—	—	—	—	—	—
IV	4a	—	—	—	—	—	—	+	+	+	—	—	—
	4b	—	—	—	—	—	+	+	+	+	—	—	—
	4c	—	—	+	(+)	—	+	+	+	+	—	—	—
V	5	—	—	—	—	—	—	—	—	—	+	+	+

+ = confluent or semiconfluent lysis; (+) = variable reaction; — = negative reaction

strains were phage sensitive in 31 and 40 per cent, respectively. The difference is explained by the fact that faecal and nose—throat strains had been isolated from samples used also for the isolation of typing phages employed in this study. The remaining Klebsiella strains were received from a different laboratory and no phage isolation was attempted from the original materials. Thus it may be concluded that for further development of phage typing, phages present in materials yielding untypable Klebsiella strains, should be isolated.

Table V shows the distribution of the isolated strains according to hospitals. Two major nosocomial outbreaks were investigated. Strains isolated from the faeces of premature infants in Hospital A belonged to phage group I in 65 per cent. Strains isolated from an enteritis epidemic occurring in the premature, infant and infectious diseases departments of Hospital B belonged in 72 per cent to phage group IV. Other strains originating from adult patients treated in various hospitals fell in 57 per cent into phage group II and in 28 per cent into phage group III.

Thus strains of phage groups I and IV might be responsible for severe cases of enteritis among premature babies and infants, while those of phage groups II and III were associated with various pathological conditions in adults.

Table IV

Distribution of Klebsiella phage types in various materials

Phage group	Phage type	Faeces	Nose – throat	Urine, ear, etc.	Necropsy	Fomites	Standard strains	Total
I	1a	63	—	—	—	—	1	64
	1b	75	15	—	3	2	—	95
	1c	6	3	—	1	1	—	11
	1d	8	—	—	—	—	—	8
	1e	9	3	—	—	—	—	12
II	2a	31	1	5	—	—	5	42
	2b	6	—	—	—	—	2	8
III	3a	14	10	2	—	—	—	26
	3b	62	11	1	3	—	4	81
IV	4a	17	—	1	—	—	—	18
	4b	74	4	6	—	1	—	85
	4c	55	7	—	—	—	—	62
V	5	14	8	4	7	1	—	34
Total typable	No.	434	62	19	14	5	12	546
	%	72.6	72.7	31.2	58.3	71.5	40.0	68.1
Total untypable	No.	164	20	42	10	2	18	256
	%	27.4	24.3	68.8	41.7	28.5	60.0	31.9
Total		598	82	61	24	7	30	802

Among the strains 68 per cent typable cultures occurred. Strains from the hospital outbreaks were typable in 73 per cent. Among those isolated from sporadic cases only 40 per cent phage sensitive cultures occurred.

Stability of phage types was estimated by performing typing of strains isolated on repeated occasions from the same persons, and also by leaving the isolated strains in the laboratory for 1 to 8 months at room temperature. It was found that, apart from some more or less definite changes, the phage sensitivity remained constant, as only intra-group alterations occurred. Inter-group changes were never observed. The intra-group type alterations were reproduced in model experiments.

Strains occurring in Hospital A during the epidemic period belonged in 83 per cent to type 1a and 1b of group I. In Hospital B, types 4b and 4c of group IV were prevalent.

From observations made at repeated phage typing of the same strains, it may be concluded that intra-group alterations occur in the course of epidemics. These alterations are probably due to phage action occurring also under natural circumstances.

Table V

Phage group distribution of Klebsiella strains according to origin

Phage group	Hospital A	Hospital B	Other	Total
Number of strains				
I	188	1	1	190
II	31	3	16	50
III	40	59	8	107
IV	—	165	—	165
V	30	1	3	34
Total typable	289	229	28	546
Untypable	167	48	41	256
Total	456	277	69	802
Percentage for typable strains				
I	65.1	0.4	3.5	34.8
II	10.7	1.3	57.2	9.2
III	13.8	25.8	28.6	19.6
IV	—	72.1	—	30.2
V	10.4	0.4	10.7	6.2
Percentage for total number of strains				
Typable	63.4	82.7	40.6	68.1
Untypable	36.6	17.3	59.4	31.9

Association between phage type and K antigens. Comparison of phage type and K antigens of the examined Klebsiella strains yielded the following results. Phage type 1a strains encountered most frequently in 1961 during the enteritis epidemic in the premature department of Hospital A, possessed K antigen 18 (6 strains from different patients). The less frequently occurring type 2 belonged to serotype K15, and one phage-resistant culture to serotype K19. Phage type 5 isolated from the enteritis outbreak in 1963 belonged to serotype K24. Phage type 4a encountered in Hospital B also contained K antigen 24. It is probable that various phage types originating from different sources, in spite of possessing a common K antigen, are not uniform. Phage type 4b strains which occurred most frequently in Hospital B, belonged serologically either to type K23, or were untypable with the used K1—30 sera. One phage type 4c strain could not be identified as belonging to serotypes 1—30. One phage type 3b strain of animal origin belonged to serotype K30. One phage type 3a strain isolated from urine was untypable with the used set of K sera.

Of the standard Klebsiella strains representing K antigens 1—30, 13 were typable with our phages:

Phage type 2a: K 1, 7, 8, 11, 12
Phage type 3b: K 13, 27, 29, 30
Phage type 1a: K 18
Phage type 2b: K 20, 26
Phage type 4c: K 22

From the results of phage typing the following conclusions have been drawn.

(1) Our phage typing scheme is suitable for tracing the spread of nosocomial epidemics caused by Klebsiellae. The method is rapid and, in spite of some variability of types, it yields reliable results.

(2) In the two hospitals so far examined two different phage types were predominating in the outbreaks of enteritis among premature infants.

(3) The observed phage type variations and antigenic lability in serological typing described by several authors were presumably due to phage activity.

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COMPARATIVE SEROLOGICAL STUDIES ON THE EFFECTIVENESS OF MONOVALENT LIVE POLIOVIRUS VACCINES GIVEN ALTERNATIVELY IN THE ORDER 2—3—1 AND 1—3—2

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Summary. In three children's homes previously non-immunized infants ranging in age from 3 to 12 months were vaccinated with monovalent live poliovirus vaccines. In one of the homes the order of types was 1, 3 and 2 (control group), in the other homes 2, 3 and 1, except for 12 children who were affected by chickenpox at the time of immunization with the type 3 vaccine. These infants received the type 3 vaccine later (2—1—3 group). The average age was 6.6, 6.0 and 7.7 months in the control group and groups 2—3—1 and 2—1—3, respectively. All the infants were given the same food. The pre-vaccination excretion of non-polio enteroviruses was 26 per cent in the control and 25 per cent in the other two groups. The respective rates of triple-negative children were 33 per cent and 31 per cent.

Seroconversion to type 2 virus was 100 per cent (20/20 and 11/11) whether this type of vaccine was the first or the last one in the series.

In certain cases some rise in anti-type 1 titre was observable as a result of vaccination with type 2 vaccine or type 2 and type 3 vaccines. The seroconversion rate for type 1 was slightly higher in the 2—1—3 group (24/24) than in the control group (10/12).

The seroconversion rates for type 3 were 75 per cent (9/12), 69 per cent (22/32), and 91 per cent in the control group and groups 2—3—1 and 2—1—3, respectively.

The types of monovalent live poliovirus vaccine are administered in the order 1—3—2 all over the world including Hungary, where children from 2 to 38 months of age have been immunized yearly since December, 1959, with the live vaccine. Different schedules were applied on two occasions only; older children were re-vaccinated according to another scheme.

As already reported [1] during the 1959—1960 vaccination campaign older children gave higher antibody responses to the type 1 live vaccine than did infants. Similar observations were made later by other authors [2, 3, 4].

When infants had been immunized with the Salk vaccine, they elicited better antibody responses to type 2 than to type 1 of poliovirus [5, 6].

It seemed thus interesting to investigate whether infants would give a higher response to type 2 than to type 1 of live vaccine, in other words, whether changing the usual scheme 1—3—2 for 2—3—1 would improve the anti-type 1 response without weakening the response to type 2, and, in addition, how this change would influence the antibody response to poliovirus 3.

Materials and methods

Vaccines. Type 1 vaccine was prepared from SABIN's strain LSc, 2ab, in the Research Institute for Poliomyelitis and Viral Encephalitides, Academy of Medical Sciences, Moscow, USSR; type 2 and type 3 vaccines were prepared from SABIN's strains P712 CH-2ab and Leon 12 a_1b, respectively, in this Institute.

Vaccination. Undiluted vaccine was transported in the liquid state to the field, where it was diluted tenfold in HANKS' balanced solution. Diluted monovalent vaccines contained 100,000 to 150,000 $TCID_{50}$ per 0.1 ml of virus. Each infant to be vaccinated according to the schedule 1—3—2 received 4 drops of type vaccine. Otherwise 2 drops were given (approximately 0.1 ml) in a spoonful of tea.

Tissue culture. Monolayers of *Macaca mulatta* kidney cell cultures were used throughout. The tube cultures were pre-incubated at 37°C for 7 or 8 days. As growth medium, HANKS' solution containing 0.5 per cent lactalbumin hydrolysate and 2 per cent calf serum; as maintenance medium, PARKER's No. 199 with 100 units of penicillin, 50 μg streptomycin and 50 μg Mycostatin per ml, were used. The inoculated cultures were incubated at 36°C.

Virus isolation. Faecal specimens suspended in 9 volumes of PARKER's 199 were centrifuged at 16,000 r.p.m for 20 minutes. The supernatants were stored at —20°C until used. After rapid thawing, 0.1 ml faecal suspension was added to each culture. Following an adsorption period of 30 minutes at room temperature, 0.9 ml maintenance fluid was added. Cultures were kept under observation for 7 days after inoculation. Specimens were considered negative if cytopathic changes failed to appear in the course of three blind passages.

Neutralization tests. Sera were inactivated at 56°C for 30 minutes and kept at —20°C until used. After thawing, the samples were centrifuged at 3500 r. p. m for 10 minutes to remove the precipitate formed accidentally. Serial dilutions were made in PARKER's 199; 0.25 ml diluted serum was mixed with an equal volume of virus, the mixtures were kept at 37°C for 2 hours and, subsequently, at +4°C overnight. Three cultures were inoculated with every mixture, 0.1 ml per tube. Subsequently, after an adsorption period of 30 minutes at room temperature, 0.9 ml PARKER's 199 was added. The virus was always re-titrated simultaneously. The virus (type 1, Mahoney Cincinnati; type 2, MEF-1; type 3, Saukett) contained 100 to 300 $TCID_{50}$ per 0.1 ml. The sera of the children of approximately the same age (all three specimens of each child), irrespective of group, were titrated simultaneously. When at least two of the three cultures inoculated with the same dilution showed no cytopathic changes, the respective serum dilution was considered positive.

Sampling and vaccination schedules are presented in Table I.

Table I

Vaccination and sampling programme

(December, 1961 — June, 1962)

Order of types	Intervention	December		January	April				May	June		Infants examined	
		2nd	3rd	4th	1st	2nd	3rd	4th	4th	3rd	4th	Number	Average age (month)
		weeks											
2,3,1	Vaccination		+	+		+						51	6.0
	Sampling (a) blood ..		+			+			+			47	
	(b) faeces..	+	+		+	+	+	+				51	
1,3,2	Vaccination		+	+		+						60	6.6
	Sampling (a) blood ..		+						+			20	
	(b) faeces..	+	+									60	
2,1,3	Vaccination		+			+			+			12	7.7
	Sampling (a) blood ..		+			+					+	11	
	(b) faeces..	+	+		+	+	+	+	++	++		12	

The infants involved in the study were kept in three children's homes. Their life conditions including nutrition were identical. They had never been vaccinated against poliomyelitis. In one of the children's homes the vaccines were administered in the order 1, 3, and 2 ("control group") while in the other two homes the suggested order of types was 2, 3 and 1. At the start of the study each group consisted of 60 infants. However, because of an outbreak of chickenpox in an isolated group, 12 infants could not be given type 3 vaccine at the due time and they received the type 3 vaccine 6 weeks after type 1 had been administered. Thus, for these infants the type schedule was 2, 1, 3.

Results

Table II shows that non-polio enteroviruses were isolated from the pre-vaccination faecal specimens of 26 per cent of the control children *(i.e.* group 1—3—2) and from 25 per cent of the children of the other groups immunized according to the changed schedule.

Table II

Non-polio enterovirus excretion before vaccination

Children's home	Number of examined infants	Excreters	
		Number	Per cent
V	21	4	19
R	41	12	29
V+R	62	16	25
K (Control)	60	16	26

As shown in Table III, the percentage of triple-negative children was 33 per cent in the control group and 31 per cent in the groups immunized in the changed order.

Table III

Triple-negative infants before vaccination

Children's home	Number of examined infants	Triple-negative infants	
		Number	Per cent
V	18	5	28
R	30	10	33
V+R	48	15	31
K (Control)	18	6	33

Fig. 1 illustrates the type 2 antibody titres. We examined three samples from each child; one was taken before the vaccination campaign, the second before the administration of the type 1 vaccine and the third sample after the last vaccination. The seroconversion rate for type 2 was satisfactory; 90 per cent and 100 per cent as judged on the basis of the second and the third samples, respectively. The same rate was achieved in the control group. The infants having no type 2 antibodies (2 out of 46 infants) in the third sample had been positive when the type 2 vaccine was administered. A similar case (1 out of 18 infants) was registered in the control group.

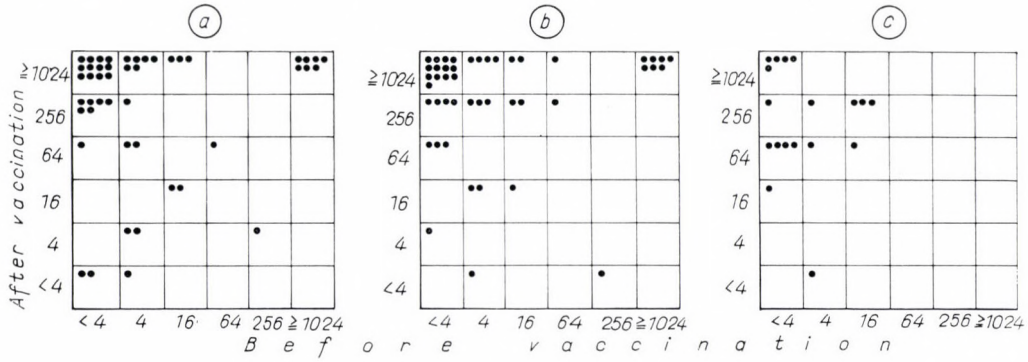

Fig. 1. Type 2 antibody titres

a) Immunization with type 2 or type 2 and 3 vaccines. Conversion rate, 19/21 = 90%. — *b)* The same children before and after immunization with type 2, 3 and 1 or type 2, 1 and 3 vaccines. Conversion rate, 20/20 = 100%. — *c)* Control group before and after vaccination with type 1, 3 and 2 vaccines. Conversion rate, 11/11 = 100%

Fig. 2 shows the changes in the type 1 antibody levels. The moderate rise in the titre of the sera of 10 out of the 46 infants after administration of type 2, or type 2 and type 3 vaccines deserves attention. In the group having received type 1 vaccine as the last dose, the seroconversion rate was 100 per cent while in the control group it was 83 per cent, in agreement with our earlier findings [1]. It should be noted that in the control group 5 out of 18 children had no type 1 antibodies in their last samples. These include infants who at the beginning of the vaccination campaign had had maternal antibodies and became negative during the vaccination period. In contrast, in the 2—3—1 group every infant had type 1 antibodies following the vaccination.

The type 3 antibody level showed a less pronounced rise than that of type 2 antibody, after immunization with type 2 and type 3 vaccines (seroconversion rate, 62 per cent) and this result improved only slightly after the last dose, *i.e.* type 1 vaccine, had been administered (seroconversion rate, 69 per cent). In the control group the seroconversion rate was 75 per cent. The best rate (91 per cent) was achieved in the group having received type 3 vaccine as the last dose because of the outbreak of chickenpox.

The pre-vaccination virus excretion is shown in Table II. In the control group virus excretion was not examined after vaccination. In the 2—3—1 group faecal specimens were collected before immunization with type 1 vaccine,

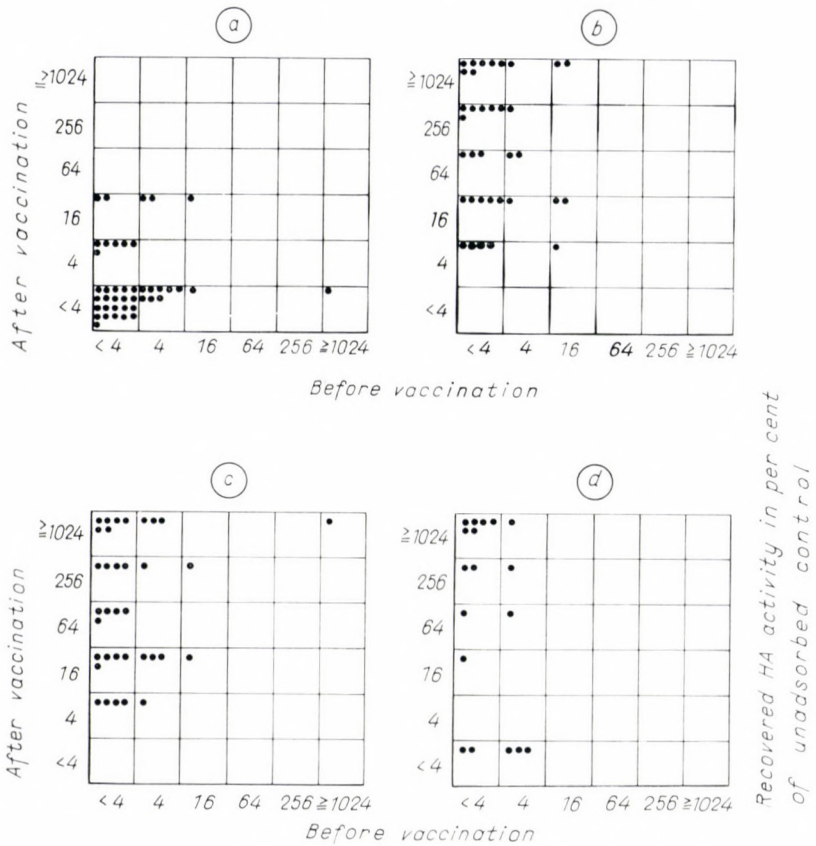

Fig. 2. Type 1 antibody titres

a) Immunization with type 2 vaccine or type 2 and 3 vaccines. — *b)* The same children before and after after additional immunization with type 1 vaccine. Conversion rate, 25/25 = 100%. *c)* The same children before and after immunization with type 2, 3 and 1 vaccines. Conversion rate, 24/24 = 100%. — *d)* Control group, see Fig. 1/c Conversion rate, 10/12 = 83%

on the 8th to 9th day, and in the period from the 11th to the 13th day thereafter. In the 2—1—3 group, the faeces was tested 3—5 days and 4 weeks after vaccination with the type 3 vaccine. Each specimen was examined separately. Before the administration of type 1 vaccine enterovirus could be isolated from none of the 104 faecal specimens obtained from 52 infants. After immunization with the type 1 vaccine, virus excretion was different in the two children's homes (73 and 33 per cent, respectively). No enterovirus other than type 1 poliovirus

was isolated. In the 2—1—3 group the type 3 vaccine virus was re-isolated from all the specimens collected on the 3rd and 5th days. Four weeks after vaccination 3 infants still excreted type 3 virus.

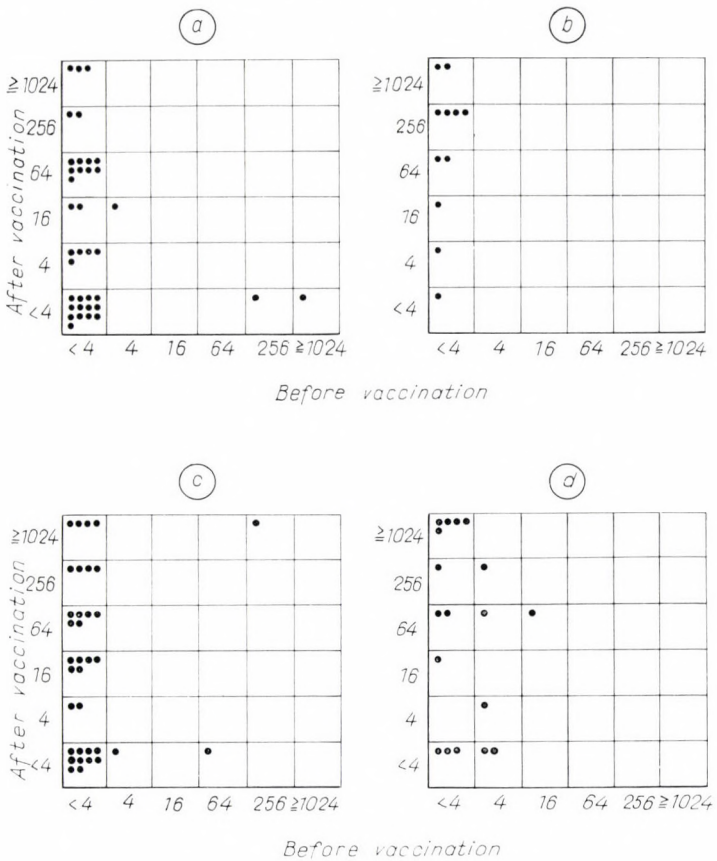

Fig. 3. Type 3 antibody titres

a) Immunization with type 2 and 3 vaccines. Conversion rate, 21/34 = 62%. — *b)* Additional immunization with type 1 and 3 vaccines of children pre-immunized with type 2 vaccine. Conversion rate, 10/11 = 91%. — *c)* Immunization with type 2, 3 and 1 vaccines. Conversion rate, 22/32 = 69%. — *d)* Control group, see Fig. 1/c Conversion rate, 9/12 = 75%

Discussion

The first aim of the present studies was to establish (1) whether or not the immune response to the type 2 monovalent live poliovirus vaccine would be weakened by beginning vaccination with this type of vaccine and, (2) whether or not the antibody response to type 1 virus would be improved by administering this type of vaccine as the last dose. There was no contact between the children's homes under study. Housing, nutrition (chiefly artificial) and age

distribution (3—12 months of age) were also comparable. In the different groups the prevaccination excretion of non-polio enteroviruses (25 and 26 per cent) and the percentage of triple-negative children (33 and 31 per cent) were also nearly identical. None of the children had been vaccinated against poliomyelitis; most of them were born after the preceding vaccination campaign, which had terminated 8 months before beginning the campaign under study. It shoud be added that, owing to the systematic vaccinations poliovirus does not circulate in Hungary, except during, and immediately after, vaccination campaigns [7]. Consequently, the antibodies demonstrated in certain prevaccination sera cannot be attributed to infection by either wild or attenuated polioviruses. In the prevaccination samples the incidence of type 2 and type 3 antibodies were the highest and lowest, respectively (see the Figs). The prevaccination antibodies failed to decrease the effectiveness of type 2 vaccine. The seroconversion rate was as high as 100 per cent, irrespective of whether this type of vaccine was the first or the last in the series. On the other hand, type 1 vaccine seemed to be more effective when given as the last instead of the first type (seroconversion, 100 per cent and 83 per cent, respectively).

It deserves attention that in certain cases (10 out of 47) the anti-type 1 titre increased after the administration of heterologous types.

The questions (1) why the immune response to type 3 vaccine was more pronounced in the control (1—3—2) group than in the 2—3—1 group and (2) how the immune response to type 3 vaccine could be improved without risking the safety of vaccination by prolonging virus circulation, need further investigation.

Experiments studying the best schedule of vaccination in larger groups of children, are in progress.

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EXCRETION OF SV-40 VIRUS AFTER ORAL ADMINISTRATION OF CONTAMINATED POLIO VACCINE

By

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Summary. SV-40 virus has been recovered from ten out of thirty-five 9—12 months old babies primovaccinated orally with type 1 attenuated poliovirus vaccine contaminated with SV-40 virus. It was found that excreters of SV-40 developed a lower antibody level against type 1 poliovirus than the non-excreters.

Although up to now there is no evidence of the pathogenicity of SV-40 in man, the investigation of this possibility appeared to be justified partly because of the cancerogenic effect of that virus in the hamster [1, 2] and partly because it has been recovered from both live and formolized vaccines, particularly polio vaccines, prepared from kidney epithelium cultures of rhesus and cynomolgus monkeys [3, 4, 5, 6].

During the winter season 1961/62 we performed experimental immunizations with live poliovirus vaccine in three infants' homes, with the aim of elucidating the correlation between the serologic response and the sequence of the individual vaccine types [7]. Later SV-40 virus was recovered from all the 3 types of the vaccine used in the experiment. The contaminant was directly demonstrable in type 1 vaccine, and in the 3rd and 4th passages of the types 2 and 3, respectively. In view of this fact we examined infants of two children's homes, where the schedule of polio vaccination was 2—3—1. The faecal samples collected after the administration of type 1 vaccine were investigated for the presence of SV-40 virus. For this purpose we used samples in which no cytopathic (CP) agent was demonstrable even when carried through 3 blind passages in rhesus kidney epithelium.

Materials and methods

Tissue culture. Stationary tube cultures prepared from the kidney epithelium of vervet (Cercopithecus aethiops) monkeys were used throughout. Until inoculation the cultures were incubated at 37°C. As growth medium Hanks' solution, containing 0.5 per cent lactalbumin hydrolysate and 2 per cent calf serum, was used. After inoculation, incubation was continued at 36° C, using a maintenance medium consisting of 94 per cent Parker's No. 199 medium and 6 per cent calf serum. The absence of SV-40 from uninoculated tissue cultures was confirmed by morphological examination.

Virus isolation. The faecal suspension which proved negative in our previous study carried out in rhesus kidney-cell cultures [7] was stored at −20°C until use in the present experiment. The material was rapidly thawed, then 0.1 ml amounts of it were distributed in tissue culture tubes, allowed to absorb for 30 minutes at room temperature and finally 0.9 ml maintenance medium was added. Each test material was inoculated simultaneously into at least 3 tubes. The infected cultures were observed over a period of 28 days, changing the medium weekly. In cases where the inoculated faecal suspension proved cytotoxic, blind passages were carried out.

Virus neutralization. Specific SV-40 hyperimmune sera were prepared in rabbits inoculated with the A-426 strain of SV-40. Polio immune sera were prepared in rabbits, using virus strains isolated and propagated in the KEM cell line [8]. Equal volumes of properly diluted type sera and the isolated CP agent were mixed and incubated for two hours at 37°C, kept in the refrigerator overnight and then allowed to stand at room temperature for 30 minutes. Tissue cultures were inoculated with 0.1 ml per tube of this material. Adsorption was allowed to proceed for 30 minutes at room temperature and then 0.9 ml maintenance medium was added to each tube. The cultures were observed for 21—28 days.

Data on the vaccination scheme and the vaccine preparation were published previously [7].

Results

A total of 56 faecal samples obtained from 35 infants aged 6—12 months were examined. SV-40 virus was isolated from 14 samples; out of 26 samples taken on the 8th or 9th, and 30 samples taken on the 11th or 13th day after vaccination SV-40 virus was demonstrable in 6 and 8, respectively. In 10 of the cases the isolate was SV-40 virus alone, whereas in 4 of the samples type 1 poliovirus was also present in spite of the fact that no CP agent was demonstrable throughout 3 blind passages in rhesus kidney epithelium (Table I).

Table I

Distribution of SV-40 positive samples according to the time of sampling

Day of sampling*	Number of samples		
	examined	containing	
		SV-40	SV-40+Polio 1
8—9	26	4	2
11—13	30	6	2
Total	56	10	4

* Days after the feeding of vaccine

Out of the 35 infants 10 excreted SV-40 virus. At the time of oral vaccination the excretors were 9 to 12 months old. Four of the infants excreted SV-40 virus at both samplings, out of them 2 SV-40 virus alone, one SV-40+ type 1 poliovirus and one SV-40+ type 1 poliovirus on the first occasion and SV-40 virus alone on the second. The remaining 6 infants excreted SV-40 virus in one sample only, 2 of them at the first sampling and 4 at the second

Out of the latter the first samples of two infants were not examined for SV-40 virus but type 1 poliovirus was demonstrable in them by inoculation into rhesus kidney epithelium cultures.

Prior to vaccination no type 1 poliovirus antibodies had been demonstrable in the sera of 7 out of the 10 SV-40 excreters. After vaccination all infants

Table II

Polio 1 antibody level before and after the vaccination of children excreting SV-40

Designation of children	Virus excretion in samples		Antibody titer to polio 1*	
	1st	2nd	before vaccination	after vaccination
P-64	SV-40	SV-40	<4	16
P-65	SV-40	SV-40	<4	64
P-68	SV-40	Negative	<4	16
P-76	Polio 1+SV-40	SV-40	1024	1024
P-89	SV-40	Negative	<4	1024
P-91	Rh Polio 1	SV-40	4	1024
P-102	Polio 1+SV-40	Polio 1+SV-40	<4	4
P-104	Negative	Polio 1+SV-40	<4	64
P-108	Negative	SV-40	<4	16

Remarks: Rh Polio 1 = type 1 poliovirus isolated in rhesus monkey kidney cultures
Negative = Negative in both rhesus and vervet monkey kidney cultures
*Reciprocals

displayed a demonstrable antibody level against the vaccine strain. In one out of 7 seronegative cases the antibody titre was 1 : 1024, in 6 cases 1 : 64 or lower (Table II). In the 17 infants who had been serologically negative prior to vaccination, and subsequently were not excreting SV-40 virus, the postvaccination titres were $\geq$ 1 : 256 in 9 cases and 1 : 64 in 8 cases.

Discussion

After the oral administration of live polio vaccine contaminated with SV-40 virus, MELNICK and STINEBAUGH [9] succeeded in reisolating SV-40 virus from the faecal samples of the vaccines, whereas SABIN [cit. 9] and MAGRATH *et al.* [4] reported on negative results. Our observations support the findings of MELNICK and STINEBAUGH [9], but the two results are not entirely comparable. At the time of oral vaccination the infants examined by us were

older than those studied by MELNICK and STINEBAUGH (9 to 12, and 3 to 6 months old, respectively). The proportion of SV-40 excreters (10/35) was also higher in our material. It ought to be mentioned in this connection that at the time of our studies the proportion of poliovirus excreters was different in the two infants' homes studied by us, being 73 per cent in one and 33 per cent in the other. Simultaneously, the percentage of SV-40 excretors was 24 and 33, respectively. We have failed to elucidate the cause of this difference, but it seemed from the findings that in children's collectives the frequency of poliovirus and SV-40 excretion is inversely related, at least when the polio-negative samples are considered.

The excretion of SV-40 virus did not counteract the development of seropositivity in children originally seronegative for type 1 poliovirus; the conversion rate was 100 per cent throughout. Nevertheless, in most cases the antibody titres were lower in the SV-40 virus excreters than in non-excreters. Among the former about half of the infants (9/17) exhibited antibody titres 1 : 256 or higher, whereas the majority of polio sero-negative SV-40 excretors had 1 : 64 or lower antibody titres to type 1 poliovirus.

Our data on virus excretion and immune response to type 1 poliovirus were suggestive of a certain interference of type 1 poliovirus and SV-40 virus. On the basis of the shortening of type 1 poliovirus excretion, the same conclusion was drawn by MELNICK and STINEBAUGH [9]. Virus interference seems to be a plausible explanation for our observation that certain faecal samples remained negative throughout 3 passages in rhesus kidney epithelial cultures, whereas in epithelial cultures obtained from vervet monkeys the simultaneous presence of type 1 poliovirus and SV-40 virus was demonstrable. From rhesus kidney epithelium cultures used for the examination of polioviruses we have succeeded in isolating SV-40 and other types of SV in vervet kidney epithelial cell cultures.

In man SV-40 antibodies are developed after 2 or 3 injections of SV-40-contaminated Salk vaccine or inactivated adenovirus vaccines [3, 4, 10]. Intranasally administered SV-40 may also propagate and evoke an immune response [11], but no demonstrable antibody formation occurred when SV-40 was administered orally. This problem has been investigated in our laboratory and the results will be published at a later time. Here we mention only that among the sera from 145 children none was capable of regularly neutralizing a 50—100 $TCID_{50}$ dose of SV-40 virus.

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LCM INFECTION OF MICE THYMECTOMIZED IN NEWBORN AGE

By

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(Received June 16, 1964)

Summary. In accordance with the observations of other authors we have found that mice thymectomized in newborn age were resistant to infection with LCM virus. As to the fate of the thymectomized and virus infected mice, three types could be observed. (1) Typical or nearly typical incubation period, development of classical symptoms and lesions followed by death. In this group of animals necropsy revealed gross residues of thymus. (2) Death after prolonged incubation (19 to 30 days). The animals developed wasting disease. (3) Survivors. The LCM virus was recovered from brain and blood samples of the survivors sacrificed in the period between the 33rd and 53rd days of the experiment.

The possible causes of late death and the immune status of the survivors are discussed on the basis of histological (brain, spleen, thymus) and haematological findings.

The thymus is known to have an important role in the development of immune mechanisms [1, 2]. Mice thymectomized at newborn age exhibit a general immunological areactivity, *i.e.* they fail to produce antibodies and they tolerate skin transplants from genetically unrelated mouse strains [3]. Neonatally thymectomized mice usually succumb to wasting disease after a survival period of 6 to 8 weeks. This syndrome is characterized by general atrophy of the lymphatic system, diarrhoea and dermatitis.

It has been found by Rowe [4], Levey [5], East [6] and the present authors[7] that LCM infection is a convenient model for the study of the development of immune mechanism in mice thymectomized in newborn age.

Traub [8, 9] observed the carrier state [10] of mice infected with LCM virus during intrauterine life. On this basis it has been suggested [11, 12] that for the fatal outcome of the intracerebral infection of fully developed mice an immune-conflict in the diseased animal might be responsible. This supposition is supported by the observation that total body irradiation [13] or treatment with cortisone or with other immunity-depressing agents [14, 15, 16] afforded resistance against intracerebral inoculation of LCM virus. The occurrence of a similar resistance was supposed to ensue in mice rendered immunologically areactive by thymectomy in newborn age.

Materials and methods

Experimental animals. Mice from the closed outbred stock of the State Blood Transfusion Service were used. The stock was tested by the usual method [17] for the absence of LCM virus.

Thymectomy. The operation was performed by MILLER's method [18]. Cannibalism of the mothers was prevented by returning the operated newborns to their nests one day later. During this period the animals were kept in an incubator. Part of the littermates served as controls. Half of the controls were subjected to sham-thymectomy, *i.e.* incision and removal of the sternum was performed leaving the thymus in place.

Examination of pheripheral blood. Leucocyte level and the changes in peripheral blood (blood films stained with May-Grünwald-Giemsa's stain) were determined at weaning. Blood was obtained from the tail vein.

Virus. Strain A-LCM, the second passage of the standard strain W. E., was kindly supplied by Dr. M. SIMON (Hungarian Army Medical Corps). In our laboratory the strain was maintained by serial intracerebral passages in mice and stored at −70°C until use. Both thymectomized and normal animals were infected intracerebrally at the age of 3 to 4 weeks with 0.03 ml of inoculum containing 100−300 LD_{50} of virus. The virus was simultaneously titrated in groups of 4 animals per dilution and the LD_{50} calculated according to REED and MUENCH.

Reisolation. Intracerebral inoculation of brain and blood samples from carrier animals was used for the demonstration of persistent virus. Brains were ground with quartz-sand, centrifuged, and the supernatant applied as inoculum. Heparinized blood samples were taken under ether anaesthesia.

Histology. Haematoxylin-eosin stained histological preparations from spleen, thymus and brain of each animal were examined.

Results

Preliminary experiments (Experiment I) were performed on three groups of mice. The summarized data from these experiments (see Table I) show that part of the thymectomized animals exhibited prolonged incubation as compared to the controls. Moreover, some thymectomized mice survived the 30th day after inoculation.

Table I

Incubation period of LCM virus infection in thymectomized and control mice
(Experiment I)

Mice	Number of mice examined	Number of mice died														
		6	7	8	9	10	12	13	14	15	16	18	19	24	28	≧30
		days after LCM virus infection														
Thymectomized	26	—	3	4	4	—	—	2	1	2	1	1	2	1	2	3*
Control	29	7	9	4	6	3	—	—	—	—	—	—	—	—	—	—

* Survivors, killed on the day indicated

The variability of the incubation period observed in operated mice suggested the possibility that thymectomy was not complete. In most cases a careful post-mortem examination of the mediastinum and the histology of the mediastinal surface of the manubrium sterni revealed the presence of some thymic residues. The possibility of some regenerative processes in such foci could not be excluded. In further experiments special care was taken to remove all possible residues of the thymus.

Table II

Incubation period of LCM virus infection in thymectomized and control mice
(Experiment II)

Mice	Number of mice examined	Number of mice died on days after LCM infection												Mortality rate
		7	8	9	10	12	19	29	30	33	47	48	53	
Thymectomized	21	—	4	1	—	1	1	1	3	3*	2*	2*	3*	11/21
Control	19	3	10	3	3	—	—	—	—	—	—	—	—	19/19

* Survivors, killed on the day indicated

In the experiment presented in Table II (Experiment II) thymectomy was performed by a more radical technique. According to their survival times, the

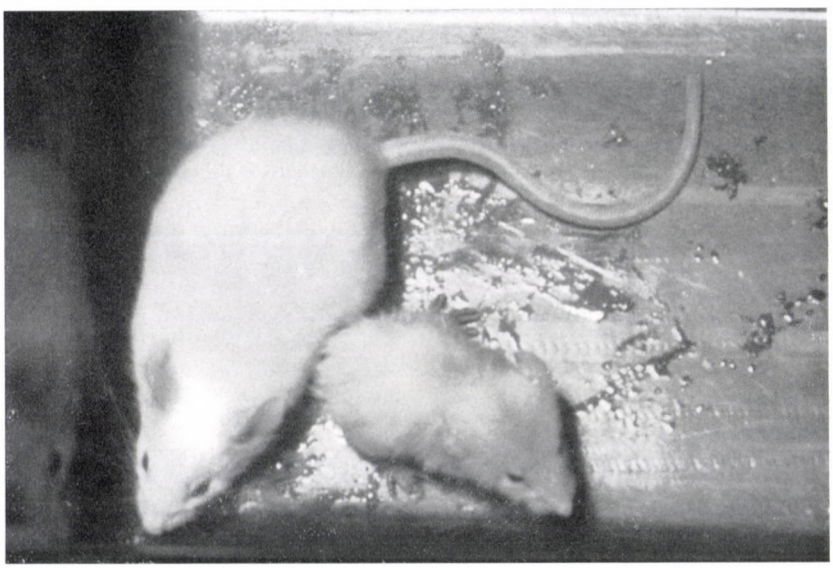

Fig. 1. Two mice from the same litter. Right: LCM virus infected thymectomized animal with typical symptoms of wasting disease. Left: control animal

infected thymectomized mice could be divided into three groups. (1) Animals dying within the normal range of incubation after having exhibited typical symptoms of LCM (6 out of 21). (2) Animals dying after prolonged incubation periods of 19 to 30 days (5 out of 21). (3) Survivors (10 out of 21). The survivors were sacrificed successively during the period from the 33rd to the 53rd day of the experiment and subjected to histological studies.

All animals from the first group (those dying within normal incubation period, after having shown typical convulsions of LCM infected mice) exhibited macroscopic thymus residues. Whereas most of the second-group animals, those dying after prolonged incubation period without typical symptoms and

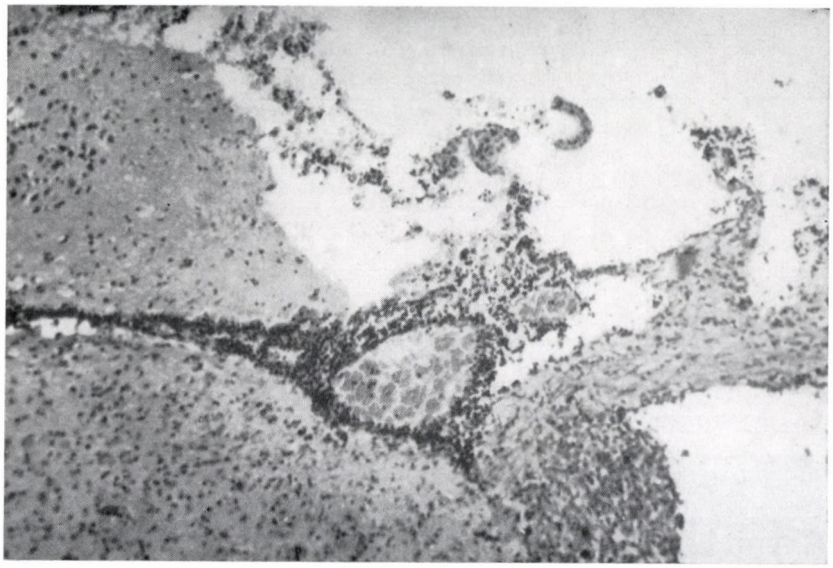

Fig. 2. Histology of the brain of a mouse died with typical LCM virus infection. Massive lymphocytic infiltration in ependyma and choroid plexus. Haematoxylin-eosin. Magnification, x70

some survivors of the third group, developed wasting disease: retardation, dermatitis as presented in Fig. 1 and spleen atrophy. These animals showed no thymic residues or only microscopic ones.

A heterogeneity (until now not explained) of the third group survivors, however, must be registered. Some of these animals having a normal appearance, clinically remained symptom-free with spleens of normal size and microscopic pattern.

Table III shows the blood counts of thymectomized and control mice. Of these data the absolute lymphocyte counts were the most suggestive; the average values being 1670 and 3740 for thymectomized and normal animals, respectively.

Persistence of the virus was invariably demonstrable in the brain and blood samples of the survivors killed after varying intervals. Nevertheless,

Table III

Changes of peripheral blood of mice thymectomized at birth

Mice	Leukocyte count (mean)	Per cent lymphocyte (mean)	Absolute lymphocyte count (mean)
Thymectomized	3100±515	53±13	1670±600
Control (not thymectomized) .	4600±615	81± 5	3740±575
Statistical significance (P)	<0.01	<0.01	<0.01

Fig. 3. Histology of the brain of a mouse thymectomized at newborn age and infected with LCM virus. Succumbed on the 30th day following infection. No signs of inflammation in ependyma and the plexuses. Haematoxylin-eosin. Magnification, x70

microscopic examination of the brain of animals showing prolonged incubation period and of the survivors revealed no pathological changes specific for LCM, although carrying virus (Figs 2 and 3).

Discussion

In agreement with the results of different authors [4, 5, 6] we have observed that neonatally thymectomized mice were resistant to LCM virus infection. The cause of death after a prolonged incubation period (19—30 days) is open to discussion. These animals might have suffered from a torpid form of LCM infection characterized by prolonged incubation and a lack of typical histological lesions in the brain. They exhibited, however, signs of wasting disease (retarded growth, spleen atrophy) with concomitant lymphopenia. The late death may thus be related to the wasting disease consequential to thymectomy.

All the thymectomized mice exhibited lymphopenia. Rowe *et al.* [4] on the other hand did not observe such a change in their thymectomized, outbred albino mice of the NIH strain. These authors therefore explained the immunological tolerance of thymectomized animals by the immune paralysis of the specific antibody producing clone and not by the general atrophy of the lymphatic apparatus. May be that some of our survivors (those without wasting symptoms, with a perfectly normal clinical appearance and spleens of normal

size and microscopic pattern) are like ROWE's animals: tolerant to LCM virus without general signs of thymectomized mice.

The brains and blood samples of the survivors killed between the 33rd and 53rd days of the experiment were regularly found to contain LCM virus. This finding was in agreement with that of ROWE *et al.* [4] and EAST *et al.* [6]. Thus these animals could be regarded as being in a condition similar to the naturally occurring or arteficially produced carrier state. The former may result from the intrauterine infection of mice, as observed by TRAUB [8, 9], the state interpreted by BURNET [10] as immunological tolerance. A similar state may, however, be achieved by the intracerebral infection of newborn mice from 0—4 days [11] and by the subcutaneous or intraperitoneal inoculation of fully developed mice. Infection with LCM virus may result in a carrier state if the animals are pretreated with cortisone [11], amethopterin [16] or total body irradiation [13], too.

Acknowledgement. We are indebted to *Dr.* E. KELEMEN (Postgraduate Medical School, Budapest) for valuable advices, to *Dr.* M. SIMON (Hungarian Army Medical Corps) for the virus strain and to *Dr.* E. CHOLNOKY (State Blood Transfusion Service) for the experimental animals. The careful and skilled technical assistance of *Miss* M. BARBIE is appreciated.

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STUDIES ON THE INTRATYPIC VARIANTS OF ECHOVIRUSES

II. THE ROLE OF CELL RECEPTORS IN THE SELECTION OF ECHOVIRUS VARIANTS

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Summary. In agreement with other investigators we have found that the haemagglutinating strains of echoviruses were losing their haemagglutinating capacity in the course of passages in certain stable human cell lines. In other experiments the existence of haemagglutinating (H^+) and nonhaemagglutinating (H^-) virions was demonstrated by us in strains of echovirus types 3, 6, 7, 12, and 19. The present experiments have shown that the loss of haemagglutinating activity in the above cultures is due to the selective inhibition of the mutiplications of H^+ virions.

Both the cell lines of human origin and the human erythrocytes possess receptors reacting exclusively with the H^+ virions. The cell and erhythrocyte receptors appear to be identical as regards resistance (both are sensitive to trypsin and formalin and resistant to periodate treatment) and the conditions of fixing virus. Similar receptors could not be extracted from primary monkey-kidney cells or cell lines of monkey-kidney origin.

The receptor substance extracted from human cell lines or erythrocytes acts only on H^+ virions, inhibiting the haemagglutination and the multiplication in human cell lines, but increasing the titre in monkey-kidney cell cultures. The titre increasing effect of receptor in monkey-kidney cell cultures might be attributed to an improved condition of virus penetration.

The possibility of isolation of H^+ echoviruses in primary monkey-kidney cell cultures from specimens of low virus contents may significantly be increased by adding human cell receptors to the cultures.

*

In the first report of this series [1] it was shown that certain strains of echovirus type 6 consist of two kinds of virion, the one being able to haemagglutinate (H^+) the other not being able to do so (H^-). Further experiments have brought evidence [2] of the existence of H^+ and H^- virions also within types 3, 7, 12, and 19. The H^+ and H^- virions are different from each other in certain further biological characteristics.

The character of a strain consisting of both H^+ and H^- virions is determined by its H^+/H^- ratio, which, however, may fluctuate in the course of passages; even selection of one or the other variant may occur.

Shifting of the H^+/H^- ratio in some direction results from differences in certain characteristics of the two kinds of virion, such as adsorption rate and ratio, length of the reproduction cycle, virus yield per cell, *etc.*, in a given system.

Several authors [3, 4, 5] have shown that in the course of passages in HeLa cell cultures haemagglutinating echovirus strains may lose their haemagglutinating activity without a decrease in their infectivity. In the light of our results we have supposed that HeLa cells contain factors inhibiting the reproduction of H^+ virions, without affecting the reproduction of the H^- virions. Among the possible factors, the cell receptors seemed to be most important in this effect, since it has been shown [6, 7] that the receptors of human erythrocytes and of some cultured cells inhibit not only haemagglutination (HA), but in certain cases even the reproduction of echoviruses.

In the present study we have attempted to reveal the possible effect of cell receptors on the H^+ and H^- virions and to establish whether cell receptors played a part in the selection of viral variants.

Materials and methods

Viruses. The H^+ and H^- variants of echovirus type 6 were separated from a strain 3887/61 isolated by us [1]. The prototype strains of echovirus types 3, 7, 12, and 19 were kindly supplied by Prof. M. P. CHUMAKOV (Moscow).

The H^+ and H^- variants of the prototype strains were separated from each other by repeated end-dilution technique. The homogeneity of the working suspensions was increased by chromatography in Ca phosphate columns [1, 8]; in the case of the H^- lines, by removing the H^+ contamination by adsorption to human O erythrocytes. The non-prototype strains used in this study had been isolated at the Virus Department of the State Institute of Hygiene, Budapest, in the years 1959—1963. They were typed by the neutralization test.

Cell cultures. Monkey-kidney primary cultures and RUZICSKA's [9] stable rhesus-kidney cell lines No. III/1 and No. XIII/12 were used in parallel experiments. Occasionally HeLa, KB, FL human amnion and HEp-2 cell cultures were also employed. Parker's 199 was used as maintenance fluid in the case of monkey-kidney cell cultures.

Infectivity tests. Three, in certain cases five, tube cultures were inoculated with each dilution of tenfold dilution series. The results were read, in general on the 7th day, in particular cases the titre was calculated on the basis of the 14th day reading. The REED—MUENCH scheme [10] was applied.

HA and HA-inhibition (HI) titrations. These were carried out in TAKÁTSY's Microtitrator apparatus [11]. Citrate-treated human O, rhesus and vervet erythrocytes were used in 1 per cent suspension. Before being used, the erythrocytes were washed three times. When the effect of pH on HA was investigated, the diluent was 0.1 *M* Tris (trishydroxymethyl-methylamine-HCl) buffer.

The methods of chromatography and that of utilizing the aqueous agar extract were described in earlier reports [1, 8].

Treatment of cells and erythrocytes. One volume of 9 per cent neutral formalin solution or two volumes of 0.01 *M* KIO_4 or 10 volumes of 0.025 per cent trypsin were added to one volume erythrocyte or HeLa-cell suspension. Formalin treatment was carried out at 4°C, KIO_4 and trypsin treatments at 37 °C. Samples were taken at intervals. The effect of periodate was suspended by adding glucose. The treated cells were centrifuged and washed with saline at +4 °C. Treatment of cells and erythrocytes with anti-cellular sera consisted of incubation with 1 : 10 diluted serum at 22 °C for an hour, and washing.

Preparation of erythrocyte and cell extracts (receptors). Our procedure was based on that of HOWE *et al.* [12]. Erythrocytes obtained from 400 ml human group-0 blood were washed six times with saline. The leucocyte layer was discarded on each occasion. One volume of packed erythrocytes was haemolyzed with nine volumes of distilled water the pH of which was adjusted to 5.5 with 0.1 *M* acetate buffer of pH 4. The haemolysate was centrifuged at 1000 *g* for 10 minutes. The sediment was washed and centrifuged with distilled water adjusted to pH 5.5 until the supernatant remained colourless. To one volume of washed and sedimented stroma two volumes of PARKER's 199 were added and, if necessary, the pH was adjusted to 7.3—7.4 with 0.1 *N* NaOH. A small part of the material thus obtained was used in this state (erythrocyte stroma), its greater part was frozen (in dry-ice-alcohol mixture) and thawed six times, stirred

with a magnetic stirrer overnight and centrifuged at 7000 *g* for 30 minutes. The extract thus obtained was termed "erythrocyte receptor". Cell stromata and receptors were prepared essentially in the same manner. The preparations obtained from cultivated cells contained the extract of 10^7 cells per ml.

To free cells from the serum present in the growth medium, the cells were washed twice with HANKS' solution one day before extraction and re-incubated in serum-free medium for a day. The cells were then suspended by EDTA 1 : 5000, washed twice with HANKS' solution, and counted before use.

Results

The effect of receptors on the HA by echoviruses. In the first experiment the cell and erythrocyte extracts were tested for HI against H^+ suspensions of different echovirus types. Four HA units were used throughout. The extract—virus mixtures were kept at 4°C for one hour and, after adding the human erythrocyte suspension, at the same temperature until reading was accomplished. The results are shown in Table I.

Table I

HI titres of cell and erythrocyte extracts against 4 HA units of virus

Origin of extract	HI titre (reciprocals) to echovirus types						
	3	6	7	11	12	13	19
Primary rhesus monkey-kidney	0	0	0	0	0	0	0
Monkey-kidney cell-line No. III/1	0	0	0	0	0	0	0
Monkey-kidney cell-line No. XIII/12	0	0	0	0	0	0	0
HeLa	16	16	32	8	32	8	32
FL amnion	64	32	128	32	128	32	64
Human "O" erythrocytes	32	16	32	8	64	8	64
Vervet erythrocytes	0	0	32	0	64	0	0
Rhesus erythrocytes	0	0	16	0	16	0	0

$0 = < 2$

Out of the extracts only those obtained from human cell lines and human erythrocytes inhibited HA regardless of the type of virus. The vervet and rhesus erythrocyte extracts were active against two types only, *viz.* types 7 and 12. Since intact vervet erythrocytes are agglutinable exclusively by the same types, the parallelism between agglutinability of erythrocytes and the HI activity of extracts is obvious.

Table I also shows that the sensitivity to the extract of different echovirus types was different.

In contrast to the extracts of cells of human origin, those of the simian cells failed to inhibit HA. After the first negative results we supposed that the sensitivity of our HI test was insufficient. To increase its sensitivity, we first wished to throw light on the course of the adsorption of the virus in the presence of different relative concentrations of virus and receptor.

For this purpose 4 HA units of echovirus type 7 were added to serial dilutions of the HeLa cell extract used also in the first experiment and the

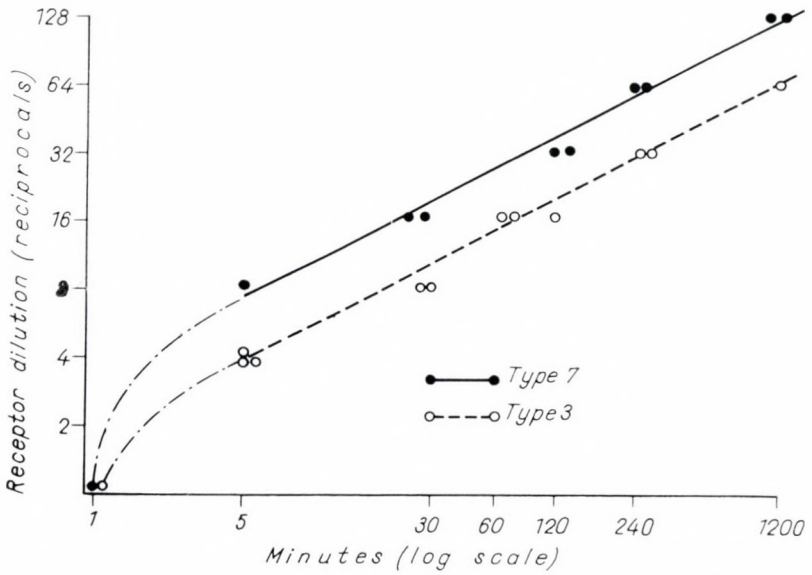

Fig. 1. Time needed for the inhibition of 4 HA units of echoviruses type 3 and type 7 in the presence of various concentrations of HeLa receptor

mixtures were kept at +4°C. Samples were taken at intervals and the time needed for the loss of haemagglutinating activity was determined. A similar experiment was carried out with echovirus type 3 (Fig. 1).

Fig. 1 shows an inverse linear relation between the logarithm of the incubation time and the logarithm of the amount of receptor needed for the total loss of HA activity. Small amounts of receptor became demonstrable when the period of incubation was prolonged. The same amount of the receptor was less inhibitory for echovirus type 3 than for the type 7 virus.

On the basis of these data we attempted to detect similar receptors in monkey-kidney cell extract by keeping extracts with 4 HA units of echovirus type 7 for 20 hours at +4 °C. No loss of HA activity was demonstrable.

Receptor concentration in the fluid and cellular fractions of HeLa cell cultures during cultivation. Before quantitative investigations it seemed reasonable to determine the possible HI activity of the rabbit serum incorporated in the

growth medium of HeLa cells. In the rabbit sera tested no HI activity could be detected. However, human, bovine (one-year-old calf) and equine sera contained inhibitors up to titres of 1 : 32—1 : 64. For this reason in the following experiment we added 5 per cent rabbit serum to the GEY's solution used as growth medium.

HeLa tube cultures were prepared by placing 100,000 cells (as exactly as possible) in each tube. During incubation cultures were taken out at intervals (10 on each occasion), the medium of the cultures was pooled and centrifuged. The supernatants were kept at —20° C until tested. The cells from the same

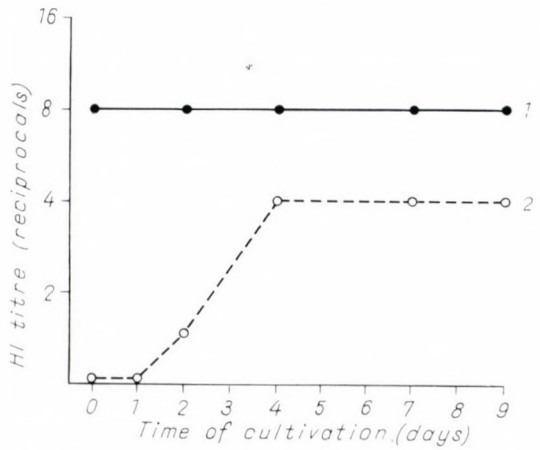

Fig. 2. HI titres of 10fold concentrated cell homogenate (1) and the growth medium (2) of HeLa cell cultures against echovirus type 7 during cultivation

cultures were suspended with EDTA, and the cell suspension, after being washed, was added to the sediment of the respective pooled medium. After centrifugation the sediment was resuspended in 1 ml of fresh medium. It was then homogenized by freezing and thawing three times, centrifuged at 3000 *r.p.m.* for 10 minutes, and the supernatants were stored at —20° C until tested. The collected materials were tested simultaneously for HI against 4 HA units of echovirus type 7. The incubation period was 2 hours.

Fig. 2 shows that during cultivation of HeLa cells the inhibitor concentration was continuously increasing in the medium while it remained at the same level in the cell homogenate. The HI titre of the medium was at least fourfold that of the cells as early as on the 4th day of cultivation. It seems likely that the inhibitor that appeared in the medium was not released by the living cells, but originated from cells detaching and disintegrating during cultivation.

Inactivation of receptor activity. On the basis of the above results we have supposed that the receptors present in the cell and erythrocyte extracts are

similar in nature to the virus-fixing receptors of intact cells and erythrocytes. To reveal the cellular location of the receptors, the following experiments were carried out.

Human erythrocytes and HeLa cells were treated with formalin, periodate or trypsin and washed; 0.1 ml of the treated red cells was added to 1.0 ml of a suspension of echovirus type 7, the mixtures were incubated at 22° C for one hour, centrifuged, and the HA titre of the supernatants was determin-

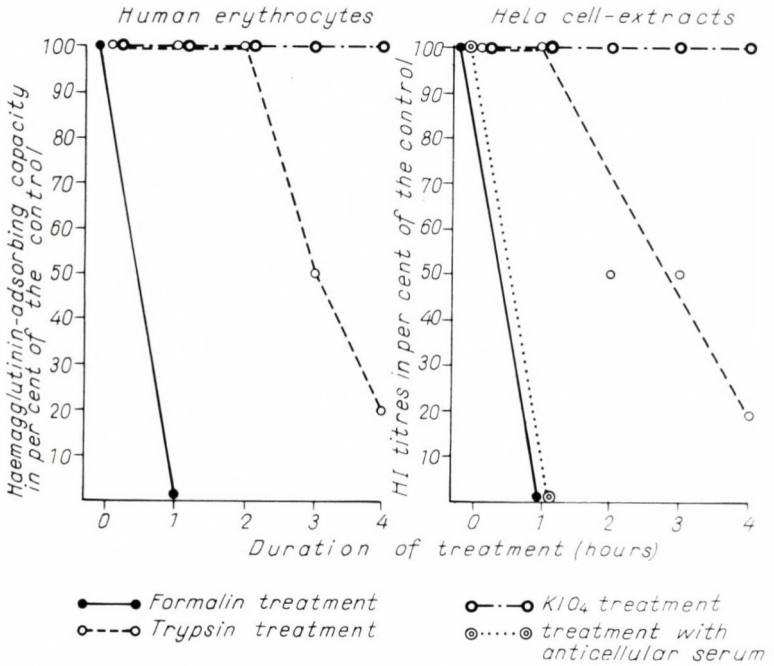

Fig. 3. Effects of formalin, trypsin, KIO_4 and anticellular serum on the echovirus-adsorbing capacity of human erythrocytes and HeLa cells

ed. The treated and washed HeLa cells were diluted in saline to make a suspension containing 5×10^6 cells per ml. This was homogenized by successive freezings and thawings, centrifuged, and the supernatants were titrated against 4 HA units of echovirus 7. In one experiment, samples of HeLa cells were treated with homologous (anti-HeLa), in another with heterologous (anti-primary-monkey-kidney) anticellular sera. The treated cell homogenates were also tested for HI activity (Fig. 3).

As shown in Fig. 3, the HeLa cell receptors able to inhibit HA due to echovirus type 7 are, like the erythrocyte receptors, located on the cell surface. Similarly to erythrocyte receptors, the HeLa cell receptors are most sensitive to formalin, less sensitive to trypsin, and are not destroyed by periodate.

Their activity can be suspended by anticellular sera. It is remarkable that formalin-treated erythrocyte and cell receptors showed some reactivation on trypsin treatment.

Factors influencing virus—cell-receptor interaction. Next the influence of pH and temperature on virus—receptor fixation was studied. For this purpose H^+ lines of echovirus types 3 and 6 were chosen, two types that behaved

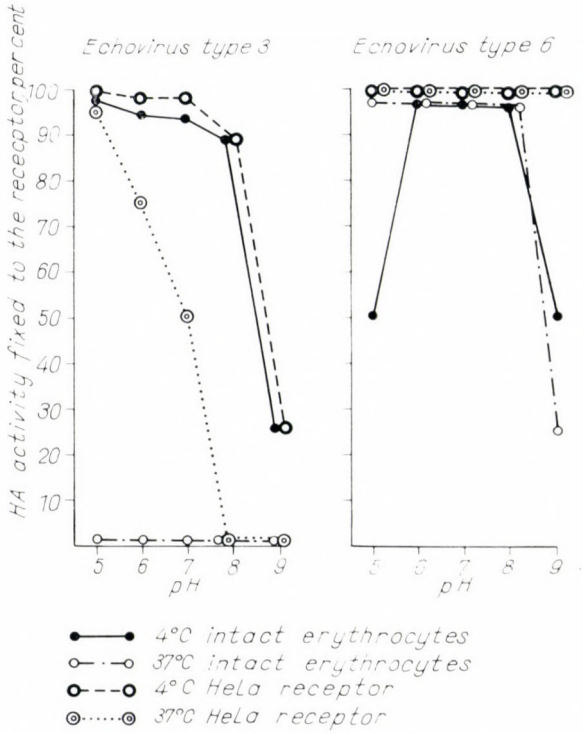

Fig. 4. Effects of temperature and pH on the echovirus-fixing capacity of intact human erythrocytes and of HeLa-cell receptors

differently in several respects. Washed human erythrocytes and HeLa cell receptors were used. One ml of the standard suspension of each of echovirus 3 and 6 having a HA titre 1 : 64 was adjusted to the desired pH with Tris-HCl buffer and 0.1 ml of washed human erythrocyte suspension or the same volume of undiluted HeLa receptor was added. Each suspension was incubated at +4°C and 37°C, for one hour. Then the suspensions containing erythrocytes were centrifuged and the HA titres of the supernatants as well as the residual titres of virus suspensions treated with HeLa cell receptors were established. The HA titres obtained were indicative of the degree of receptor-virus fixation (Fig. 4).

Fig. 4 shows that adsorption of virus to the eryhrocyte receptors *(i.e.* HA reaction) required approximately the same conditions as the reaction of the soluble HeLa receptors with the virus. Echovirus type 3 was fixed at the highest ratio to both cell-fixed and soluble receptors at +4°C and pH 5—7; at 37°C on the other hand, this virus was not fixed to erythrocyte receptors; at that temperature it was bound to HeLa receptors but only in an acidic environment.

The fixation of echovirus type 6 is, on the other hand, independent of temperature and even the pH of the medium is of no importance, except in the case of the erythrocyte receptor.

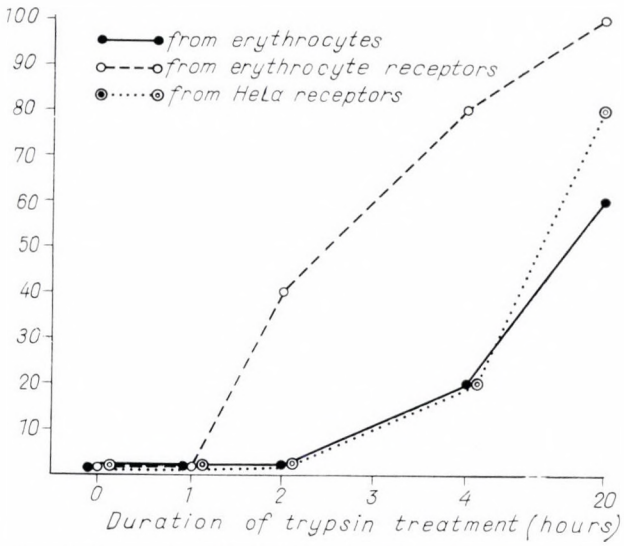

Fig. 5. Reactivation with trypsin of the HA activity of echovirus type 7 fixed to intact erythrocytes, erythrocyte- or HeLa-cell-receptors

The H^+ variants of echovirus types 7 and 12 behave like type 6 whereas those of type 19 imitate type 3.

The H^+ virions of type 6, 7 and 12 echoviruses can be separated from the receptor by trypsin treatment. An experiment of this type carried out with our echovirus type 7 standard suspension (HA titre, 1 : 128) is described in the following.

To 1 ml of virus 0.1 ml of washed human erythrocytes, to another sample the same volumes of erythrocyte- and HeLa-receptor, respectively, were added. The mixtures were incubated at 37°C for 2 hours, then an equal volume of 0.025 per cent trypsin solution was added and incubation at 37°C was continued. From this time on samples were tested for HA at intervals. Titration was carried out at +4°C (Fig. 5).

The virus was recovered from each of the receptor preparations tested.

The effects of cell receptors on the reproduction of echovirus variants. In accordance with several authors [3, 4, 5] we found in earlier studies that haemagglutinating echoviruses, when cultivated in HeLa cells, lost their HA activity. We have shown that H^+ lines do not propagate in HeLa cultures; thus from cultures consisting of H^+ and H^- virions the H^- variant will be selected as early as in the first few passages in HeLa cells. In contrast, both H^+ and H^- variants propagate in monkey-kidney cell cultures. Therefore, we chose the latter cultures for the experiments in which the influence of HeLa and monkey-kidney cell extracts on the multiplication of the two variants was investigated. These experiments were repeated several times. The H^+ and H^- variants of types 3, 6, 7 and 12 were tested.

Three parallel tenfold dilution series were prepared from each of the variants under study. The diluents in the three titration series were (i) PARKER's 199 (control), (ii) HeLa cell extract containing 4 HI units per ml, and (iii) identically diluted monkey-kidney cell extract showing no HI activity. Three monkey-kidney cell cultures were inoculated with each dilution, 0.1 ml per tube. (The cells had been washed twice with HANKS' solution before inoculation.) The maintenance fluid was PARKER's 199, added after 2 hours incubation with the inoculum at 37 °C. The experiments were evaluated on the basis of 7th-day readings when further incubation would not have influenced the result.

Table II shows that the titre of the variants was not influenced by the monkey-kidney cell extract. The HeLa cell extract, on the other hand, increased

Table II

Effects of monkey-kidney cell and HeLa cell receptors on the infective titres of H^+ and H^- variants of echoviruses

Type of virus	Variant	log CPD_{50}/0.1 ml		
		control	in the presence of	
			monkey-kidney cell extract	HeLa cell receptor
Echo 3	H^+	4.75	4.75	6.75
	H^-	6.50	6.00	6.50
Echo 6	H^+	2.50	2.50	6.50
	H^-	4.50	5.00	4.50
Echo 7	H^+	4.50	4.50	7.00
	H^-	3.50	3.50	3.50
Echo 12	H^+	4.00	3.50	6.00
	H^-	6.50	6.50	6.50

the titres of each of the H^+ variants to 100—10,000fold the corresponding control values, while it did not influence the multiplication of the H^- variant.

To obtain more information about the titre-increasing effect of the HeLa-cell receptor substance on the H^+ variants, first the highest dilution of the HeLa cell receptor was determined in the presence of which an increase in titre was still demonstrable. For this purpose a twofold dilution series was prepared in PARKER's 199 from the HeLa receptor prepared as described under "Materials and methods". From every dilution 0.9 ml was placed in each of four monkey-kidney cell cultures immediately after washing the cells with HANKS' solution. Then 0.1 CPD_{50} of the H^+ variant of echovirus type 3 was added in a volume of 0.1 ml. As a control, tube cultures prepared without adding receptor to the medium were inoculated with the same dose of the virus. On the 7th day of incubation none of the cultures except those containing receptor in the medium showed CP effect. The titre of the receptor preparation was expressed in terms of the dilution containing the smallest quantity in the presence of which at least two of the four tubes showed the specific CP effect. In this term the titre of the receptor preparation tested was four times higher than its HI titre as determined against 4 HA units of the H^+ line of echovirus type 3 after 20 hours incubation.

Subsequently, extracts of human, rhesus and vervet erythrocytes and those of KB, Detroit-6, HEp-2 and FL amnion cells were tested in similar, but not quantitative, experiments. The titre-increasing effect of the undiluted extracts was examined in three monkey-kidney tube cultures, each infected with 0.1 CPD_{50} of H^+ variant of echovirus type 3. CP effect was observed in every tube except the controls and in the tubes containing rhesus or vervet erythrocyte extracts.

An experiment of the same type was carried out with the H^+ variant of echovirus type 12 and extracts of human, vervet and rhesus erythrocytes. All extracts proved to increase the titre, thus showing a close parallelism between titre-increasing effect and HI capacity.

Attemps to elucidate the phenomenon of titre increase. We supposed that the addition to monkey-kidney cells of receptor substance derived from HeLa or other human cells acted by improving the conditions of virus adsorption. To do so, however, the receptor itself must be able to be adsorbed to the cells originating from a different species. We attempted to prove the adsorption of the receptor by the following experiment.

A HeLa-cell extract with a HI titre of 1 : 6 was added to washed monkey-kidney cell cultures, 1 ml per tube. The cultures were incubated without rotation at 37°C. During incubation the medium of three cultures was pooled, the cells were washed three times with HANKS' solution and then homogenized in 1.0 ml of PARKER's 199 by freezing and thawing. The pooled media and the cell

Table III

Detectability of HeLa cell receptor in primary rhesus-kidney cell cultures treated for different periods with receptor

Duration of receptor treatment	HI titres* to echovirus type 7 in	
	medium	cell-homogenate**
0 minute	6	0
30 minutes	6	1
60 ,,	6	1
120 ,,	4	2
240 ,,	4	3
24 hours	4	4
48 hours	3	0
Controls		
(a) Untreated monkey-kidney cell cultures	0	0
(b) Receptor after 48 hr at 37°C	6	

* Reciprocals. ** × 3 concentrated homogenates

homogenates were tested for HI against 4 HA units of echovirus type 7 (Table III).

The HeLa cell receptor was adsorbed by the "heterologous" monkey-kidney cells, as proved by the continuous increase in the concentration of the receptor substance in the cell fraction and its decrease in the medium. However, after 48 hours HeLa cell receptors could no longer be demonstrated in the monkey-kidney cell fraction.

Further experiments were carried out to obtain information on the possible significance of time factors in the increase of titre. Three parallel titrations were carried out in monkey-kidney cell cultures. In the first series the cells were pre-incubated for 2 hours in the presence of 2 HI units/ml HeLa cell receptor and infected after being washed twice with HANKS' solution. In the second series the cells were treated with the same amount of HeLa receptor for 2 hours, after infection. The third series included untreated cultures. The cultures were infected with 0.1 ml of echovirus 3 H^+ dilutions each, then the tubes were incubated at 37°C for one hour, the unadsorbed virus was removed by washing, and 1 ml of medium was added to each culture. An identical experiment was carried out with the receptor extracted from human O erythrocytes. The cell cultures were observed for 14 days (Table IV).

Both HeLa and human erythrocyte receptors increased the titre of the echovirus type 3 H^+ line, irrespective of whether the cells were treated before or after infection. In the former case, however, the development of the final titre was slightly delayed. The fact that the titre was increased by the receptor even if it had been added after the infection speaks against the assumption that the increase in titre is due to an improvement in virus adsorption.

Table IV

Effects before and after inoculation of treatment with HeLa and erythrocyte receptors on the infective titre of the H^+ variant of echovirus type 3 in rhesus-kidney cell cultures

Treatment of monkey-kidney cell cultures	Final titre attained on day	Infectivity (log CPD_{50}/0.1 ml)
Nil	6	4.75
Pre-treatment with HeLa receptor	9	7.0
Human "O" erythrocyte receptor	9	6.5
Post-treatment with HeLa receptor	6	6.25
Human "O" erythrocyte receptor	6	6.5

Practical utilization of the titre-increasing effect of "heterologous" receptor. We supposed that adding HeLa receptor to monkey-kidney cell cultures would render the cultures more sensitive to the haemagglutinating echoviruses in isolation experiments. To prove the supposition, we chose from the material

Table V

Titration of 16 faecal suspensions containing echoviruses in untreated and HeLa-receptor-treated monkey-kidney cell cultures

Designation of faecal suspension	Type of isolated echovirus	Infective titre (log CPD_{50}/0.1 ml) in monkey-kidney cell cultures	
		untreated	HeLa-receptor-treated
636/1961	1	2.0	1.5
608/1961	2	2.0	1.5
620/1961	2	1.0	1.0
771/1961	2	2.5	2.5
41/1961	4	1.5	1.5
665/1961	5	2.0	2.0
33/1961	6	<1.0	1.5
676/1961	6	<1.0	3.0
1167/1961	6	1.0	>3.5
1232/1961	6	1.0	>3.5
1463/1961	6	1.5	>3.5
2060/1961	6	1.0	>3.5
696/1961	7	1.5	2.5
715/1961	7	<1.0	2.0
690/1961	12	1.0	>3.5
4763/1960	19	<1.0	>3.5

of the Virus Department of the State Institute of Hygiene 16 faecal specimens which had yielded various echovirus strains. Some of these strains did while others did not agglutinate erythrocytes. The faecal suspensions had been stored at −30 °C since their arrival. All specimens were titrated both in untreated monkey-kidney cell cultures and in those treated with 0.1 ml of HeLa receptor containing 2 HI units. The results are shown in Table V.

The faecal specimens that contained echovirus types 1, 2, 4 or 5, *i.e.* non-haemagglutinating strains, yielded comparable titres in the parallel titrations. In contrast, the specimens containing the haemagglutinating strains of types 6, 7, 12 or 19 gave significantly higher titres in the pre-treated cultures than in the controls. It is of interest that all the type 6 strains isolated in untreated cultures proved to be of H^- character whereas the strains of the same type isolated from the same specimens in pre-treated cultures agglutinated erythrocytes.

Discussion

The present experiments have shown that the erythrocytes agglutinable by certain echoviruses, namely human O erythrocytes for all agglutinating strains, and rhesus and vervet erythrocytes for types 7 and 12, as well as the cell lines of human origin, contain receptor substances capable of fixing the H^+, but not the H^-, variants of echoviruses. As a result of the fixation to the receptors dissolved in the medium, the H^+ virions lose their capacity of agglutinating erythrocytes and of propagating in cells possessing the similar receptor. On the other hand, in cells lacking this receptor on their surface the strains of H^+ character show an enhanced infectivity when the receptor is added.

Fixation of the virus to the dissolved receptor derived from HeLa and other human cells is in many respects similar to the fixation to the erythrocyte receptor *in situ*. For the fixation to the dissolved cell receptor of the echoviruses which agglutinate erythrocytes only at +4°C *(e.g.* echovirus type 3), +4°C is the most favourable temperature, while for the types *(e.g.* echovirus type 6) haemagglutinating at 37°C as well as at +4°C, fixation to the dissolved receptor is not temperature-dependent. The pH dependence of the HA activity runs parallel with that of the fixation to the cell receptor.

The receptor is not injured by KIO_4, but is inactivated by formalin and decomposed by trypsin. The receptor treated with formalin for a short period of time is reactivated by appropriate trypsin treatment. Trypsin can separate the virus from the receptor.

We have used the designation "receptor" though the receptor reacting exclusively with the H^+ echoviruses (H^+ receptor) is not identical with the so-called enterovirus receptors of the susceptible cells [13, 14, 15]. On primary monkey-kidney cells we could not demonstrate the H^+ receptor, although

these cells are able to adsorb H^+ and H^- virions to about the same degree. Consequently, the H^+ receptor is not essential for the adsorption of echoviruses to the susceptible cells. On the contrary, its presence in some human cultures may definitely inhibit the reproduction of H^+ virions. This phenomenon appears to be analogous to the so-called "phenomenon of rejected particles" described by FENWICH and COOPER [16] for polioviruses. This means that, at temperatures above 23°C, 60—80 per cent of the adsorbed poliovirus particles separate from the cells and, being associated with certain substances of cellular origin, are no longer able to become adsorbed and thus lose their infectivity.

Our results are in several respects inconsistent with certain literary data. Before discussing the divergencies we wish to point out some factors which seem to have a decisive role in them.

(i) In all the earlier studies concerning the HA by, and HI receptor for, echoviruses, haemagglutinating strains containing also H^- virions had been used, so that the results were influenced by the relative amount of H^- particles.

(ii) In the course of the preparation of receptor substances and in the experiments carried out with these substances [17, 18, 19, 20, 21] the fact that human and certain animal sera contain echovirus inhibitors was disregarded. Consequently, if the growth and/or maintenance medium of the cell culture contained serum, cell-adsorbed serum inhibitor may have been present in the cell extracts; thus the inhibition due to the serum factor and that exerted by the receptor may have been summed up.

(iii) When the receptor effect is investigated, the use of human cell lines may be misleading, since in such cells the multiplication of H^+ virions is inhibited by the cells' own receptors.

(iv) For infectivity titration the agar-overlay technique is not adequate, since according to our investigations [1, 2] the agar polysaccharide may inhibit the H^- variants, *i.e.* reduce the titre of heterogeneous (H^+ and H^- containing) virus populations.

The results of PHILIPSON and BENGTSSON [6] and of PHILIPSON *et al.* [7] agree with our findings in several essential respects. The most significant divergencies are that these authors detected HI substances in monkey-kidney cells, too, and that their haemagglutination-inhibiting receptors (prepared from human erythrocytes, HeLa, KB, or monkey-kidney cells) inhibited virus multiplication. In the latter case, however, the paper leaves some doubt as to the kind of culture (HeLa or monkey-kidney) used in the infectivity-inhibition experiments carried out by the plaque technique. As regards the receptors demonstrated in monkey-kidney cells, they might have been serum inhibitors. According to the same authors the HI receptors were inactivated by chymotrypsin or papain, but not by trypsin. However, they treated the preparations for 40 minutes only while in our experiments the trypsin effect took longer to develop (see Fig. 5).

TSILINSKY [22, 23, 24] and TSILINSKY and LEVASHEV [25] reported the isolation of enterovirus inhibitors from the cellular fraction and medium of stable cell lines. These substances were active against both haemagglutinating and non-haemagglutinating enteroviruses. It cannot be decided, however, whether or not the above mentioned factors *(e.g.* serum inhibitor) had influenced the outcome of these experiments.

Our finding that HeLa or human erythrocyte receptors increase the sensitivity to H^+ echoviruses of monkey-kidney cell cultures up to the 10 000 fold of the control cultures is of special interest; such observations have not been published in the literature.We have shown that the H^+virions are adsorbed onto untreated monkey-kidney cells, but only about 1 per cent of the virions is able to penetrate such cells and thus to initiate infection.The adsorbed virions which fail to penetrate into cells retain their infectivity for one or two hours as shown by the titre-increasing effect of the receptor added to the cultures two hours after infection. We suppose that the HeLa and erythrocyte receptors increase the titre by promoting the penetration of virions of full infectivity; only a small proportion of these penetrates into cells in the absence of receptor.

Adding heterologous (HeLa, human erythrocyte) receptors to monkey-kidney cell cultures seems to increase the possibility of isolation of haemagglutinating echoviruses from specimens of low virus contents but does not interfere with the isolation of non-haemagglutinating echoviruses. Finally, the receptor appears to moderate the toxic effect of certain faecal specimens.

The special H^+ receptors of the stable cell lines of human origin play an important role in the selection of variants from inhomogeneous virus strains and may explain why the haemagglutinating echoviruses lose their HA capacity during propagation in stable human cell lines, *e.g.* in HeLa cultures [3, 4, 5].

It is only natural that other factors, such as serum inhibitors in the medium, the actual distribution of different (H^+ and H^-) virions in the inoculum and the weaker adsorption and penetration capacities of the H^+ virions, may also influence the selection even in the most favourable cell cultures.

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EXAMINATION OF THE SUSCEPTIBILITY TO VIRUS INFECTION OF HETEROPLOID MONKEY-KIDNEY CELL STRAINS

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Summary. The susceptibility to virus infection was studied of 7 monkey-kidney cell strains developed in our laboratory.

The wild poliovirus strains attained similar titres in both the cell strains and the primary monkey-kidney cell cultures, while the attenuated poliovirus strains gave lower yields in the former than in the latter cells.

Established cell strains were found to be superior to primary monkey-kidney cultures in view of both their virus ranges and their usefulness for the detection of different viral agents in clinical samples. The possibility of using the cell strains for viral diagnostics is discussed.

The establishment of 7 different cell strains (MCS) from primary monkey-kidney cell cultures has been reported previously [1, 2, 3]. The present paper gives an account of studies concerning the sensitivity of the cell strains to infection with different viruses.

Materials and methods

Tissue culture. Primary monkey-kidney (PMK) cultures were prepared according to YOUNGER [4]. Test tubes were seeded with 2×10^5 cells in 1 ml of medium each and the cultures incubated at 37°C. The cell monolayer had developed within 6 to 7 days. The growth medium consisted of HANKS' solution [5] containing 0.5% lactalbumin hydrolysate and 2 per cent calf serum, while PARKER's synthetic medium [6] was used for maintenance.

Since their establishment our monkey-kidney-cell strains (MCS) had undergone an average of 120 to 200 passages. Their growth and morphological characteristics remained unimpaired throughout. Stock cultures were prepared in 2 l. Roux flasks. From these stocks the cells were obtained by EDTA treatment (dilution $1 : 5 \times 10^3$) and used for the preparation of stationary tube cultures. MCS cultures were incubated at 37°C. The input cell count per tube was 1×10^5 per tube for strain III/1 and 5×10^4 for the other strains. The development of a confluent cell layer was complete in 4 to 6 days with strain III/1 and in 2 to 3 days with the other strains. All MCS were grown on medium PM as described previously [1] and PARKER's synthetic medium was used for their maintenance.

Viruses. The viruses used were Mahoney, MEF-1, Saukett, LSc 2ab, P712, Ch 2ab, Leon $12a_1b$ poliovirus strains; prototype strain of 1, 2, 3, 5, 7, 8, 11 and 14 adenovirus types; A2, A4, A6, B3 Coxsackie virus types; 3, 6, 7, 11, 12, 13, and 19 echovirus types; and 1, 2 and 3 parainfluenza types. In addition, one strain each of polyoma and vaccinia viruses were tested.

Titrations. Tenfold dilution series were prepared from the appropriate virus. The cell monolayers were inoculated with 0.1 ml of the individual dilutions each. After 30 minutes of incubation 0.9 ml of maintenance medium was added to each tube. Titration was performed in 10 tubes per dilution and the 50 per cent end-point was calculated according to REED and MUENCH [7].

Virus isolation. A total of 25 faecal samples from healthy children of 3 to 20 months of age was examined. The samples were collected during the country-wide live poliovirus vaccination program carried out from December, 1962, to March, 1963. The samples were prepared for examination according to the method described previously [8] and stored at −20°C until tested.

Inoculation of the cell monolayers was made with 0.2 ml of the samples each and 30 minutes were allowed for the adsorption of the virus at 37°C. This was followed by adding 0.9 ml of PARKER's medium to each tube. A minimum of 3 transfers was performed with every sample, to eliminate possible toxic effects.

Identification of the isolated agents. When the cytopathic effect persisted for 2 consecutive passages, the material was considered to contain a virus. Identification of the agent was attempted by the type sera polio 1, 2, 3; coxsackie A7, A9, B1—6; echo 1 to 26; and adeno 1 to 7, 11 and 14 to 17, prepared by the Diagnostic Laboratory of the Virus Department as described earlier [8].

Results

The sensitivity of MCS to virus infection was examined by three different methods (1). Parallel titrations of poliovirus strains were performed on PMK and MCS cell cultures and the titres compared. (2) Faecal samples positive and negative in PMK were re-examined on MCS cultures in order to compare the efficiency of isolation in the two different systems. (3) The possibility of growing on MCS of viruses which had failed to multiply on PMK was studied.

(1) *Growth of poliovirus strains on MCS.* Parallel titrations were carried out on PMK and the actually tested MCS cultures using identical virus dilutions for both systems. The poliovirus strains examined by this method were, Mahoney, MEF-1, Saukett, LSc 2ab, P 712 Ch 2ab and Leon 12 a_1b. The results obtained are presented in Table I.

Table I

Yields of different poliovirus strains in the individual cell strains as referred to those in primary monkey-kidney cells

		Poliovirus strains					
		Mahoney	MEF-1	Saukett	LSc 2ab	P 712 Ch 2ab	Leon 12a_1b
Titres (-log) in primary monkey-kidney cultures		7.2	7.1	7.5	7.0	6.8	7.1
		Titre differences (log) to those obtained on PMK					
Cell strains	I/1/a	0	0.76	0	2.92	1.74	0
	VIII/1/a	0	0	1.0	0.44	2.0	2.74
	II/1/a	0.45	0.44	1.0	2.16	1.18	1.10
	III/1	0.63	0.88	0	1.50	2.24	0.50
	XIII/11	1.00	0	0.76	2.24	2.00	1.74
	XIII/12	0	0	0.55	1.10	0.78	0
	XI/1/a	0	0	0	0.85	1.58	2.18

The "wild" strains of poliovirus gave 0 to 1.0 log. unit lower average yields in MCS than in PMK cultures. For the attenuated strains this difference varied between 0 to 2.9 log. units. Out of the MCS tested, strain XIII/12 was the one usually producing virus yields comparable to those found in PMK.

Comparative titrations have been carried out since 1960 on PMK and strains I/1/a and VIII/1/a of MCS, using Mahoney and LSc 2ab polioviruses.

As demonstrated in Fig. 1 the sensitivity of the cell strain I/1/a exhibited an increasing and that of strain VIII/1/a a decreasing tendency during the period of observation.

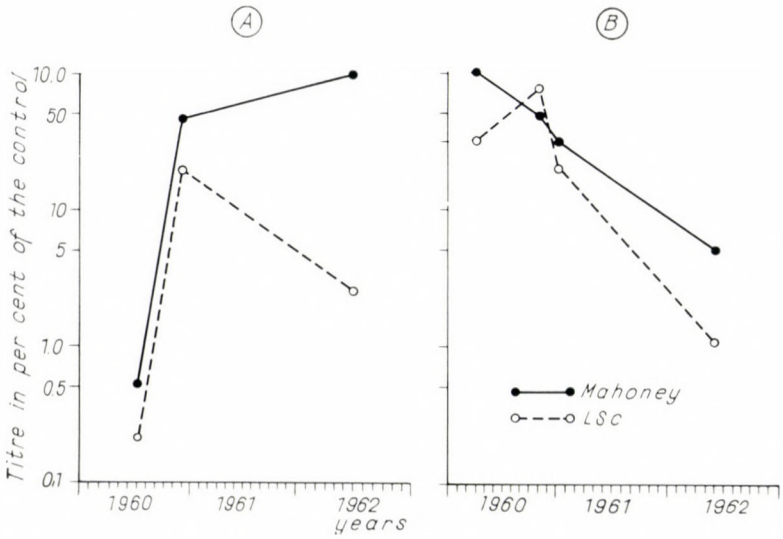

Fig. 1. Changes in the susceptibility of cell strains I/1/a (A) and VIII/1/a (B) to poliovirus infection during the period 1960—1962

(2) *Isolation experiments.* Out of the 25 faecal samples specified above 15 yielded cytopathogenic agents and 10 did not. The test was performed on PMK cultures by the staff of the Diagnostic Laboratory. The original samples were re-examined by us on different MCS. All the 15 positive samples were found to be positive also in the different MCS cultures used. Out of the 10 samples negative on PMK, some yielded cytopathogenic agents when inoculated to strains I/1/a, VIII/1/a, II/1/a and XI/1/a of MCS. Some of the agents were transferable later on PMK too, some others could, however, be maintained only on the cell strain on which they had been isolated. Pertaining data are given in Table II.

Samples 13/63 and 19/63 yielded on cell strain I/1/a two cytopathogenic agents identified later as adenoviruses. None of them could be grown on PMK cells. Cytopathogenic agents were isolated from samples 25/63, 31/63, 49/63

Table II

Examination on cell strains of faecal samples

Faecal sample No.	I/1/a			VIII/1/a			II/1/a		
	A	B	C	A	B	C	A	B	C
7/63	—	—	.	—	—	—		—	.
13/63	+	—	adeno	—	—	.	—	—	.
19/63	+	—	adeno	—	—	.	—	—	.
25/63	?	—	.	—	—	.	—	—	.
31/63	?	—	.	—	—	.	—	—	.
49/63	?	—	.	—	—	.	—	—	.
55/63	+	+	polio	+	—	polio	+	—	polio
61/63	—	—	.	?	—	.	—	—	.
85/63	—	—	.	—	—	.	—	—	.
115/63	—	—	.	—	—	.	—	—	.

A = result of isolation. B = transferable to primary monkey-kidney cells. C = result

and 61/63, too. Out of them the first three agents were obtained in strain I/1/a cells and the last one in strain VIII/1/ a cultures. None of these agents could, however, be maintained for more than 2 consecutive passages. The faecal sample 55/63 yielded a typical poliovirus in I/1/a, VIII/1/a II/1/a and XI/1/a strains of MCS. A cytopathogenic agent still under continuous passage has been isolated from sample 85/63 on MCS cultures. The agent failed to multiply in PMK and was not identifiable with any of the specific immune sera used in this study.

The survival of MCS is generally shorter than that of PMK, thus after 10 days' microscopic examination of the former yields questionable results. This drawback is absend in strain III/1 which survives in good condition for 3 weeks or more. It seemed, therefore, reasonable to perform some additional studies with the latter cell strain. Results obtained are presented in Tables III and IV.

Faecal samples yielding positive results on PMK were equally positive on MCS III/1. Out of those negative on PMK, samples 476/63, 477/63 and 479/63 were found to contain cytopathogenic agents when inoculated to MCS III/1. None of these agents could be identified satisfactorily.

negative in primary-monkey kidney cells

III/1			XIII/11			XIII/12			XI/1a		
A	B	C	A	B	C	A	B	C	A	B	C
—	—	.	—	—	.	—	—	.	—	—	.
—	—	.	—	—	.	—	—	.	—	—	.
—	—	.	—	—	.	—	—	.	—	—	.
—	—	.	—	—	.	—	—	.	—	—	.
—	—	.	—	—	.	—	—	.	—	—	.
—	—	.	—	—	.	—	—	.	—	—	.
—	—	.	—	—	.	—	—	.	+	+	polio
—	—	.	—	—	.	—	—	.	—	—	.
—	—	.	—	—	.	—	—	.	+	—	not identified
—	—	.	—	—	.	—	—	.	—	—	.

of identification

(3) *Growth of different viruses on MCS.* Three parallel passages were performed on MCS and PMK cultures using viruses growing poorly in the latter system. Results are given in Table V.

All MCS were found to support the growth of prototype strains 1, 2 and 5 of adenovirus. Prototype strains 3 and 7, however, multiplied only in cell strains I/1/a, VIII/1/a and in PMK cells. Adenovirus type 8 prototype strain could be recovered after 2 "blind" passages in cell strains I/1/a and II/1/a only. Of the other prototype strains of adenovirus, types 11 and 14 could be grown on MCS II/1/a and VIII/1/a, II/1/a, respectively.

Only the A4 type among the A2 A4 and A6 types of coxsackieviruses exhibited some vague cytopathogenic effect in cell strains VIII/1/a and II/1/a. Coxsackie B3 strain could be maintained only in cell strain III/1.

Prototype strains 3, 6, 7, 11, 12, 13 and 19 of echoviruses could be grown on cell strains III/1 and XIII/12.

The parainfluenza type strains 1, 2 and 3 were grown successfully on cell strain II/1/a. Cell strains III/1 and XIII/12 supported the growth of parainfluenza type 3.

The growth of vaccina virus was examined only in cell strain III/1. The virus grew well in this system.

Table III

Efficiency of virus isolation in cell strain III/1

Faecal sample		Result of isolation in cell strain III/1
No.	Virus isolated by the Diagnostical Laboratory	
470/63	polio 1	positive
488/63	polio 1	,,
492/63	polio 2	,,
500/63	adeno 3	,,
502/63	polio 3	,,
508/63	polio 3	,,
509/63	polio 3	,,
510/63	polio 2	,,
524/63	polio 3	,,
534/63	polio 2	,,

Table IV

Examination on cell strain III/1 of faecal samples negative on primary monkey-kidney cultures

Faecal samples No.	Result in cell strain III/1	Transferable to primary monkey kidney cultures
471/63	Negative	—
472/63	Negative	—
473/63	Negative	—
475/63	Negative	—
476/63	Positive	yes
477/63	Positive	yes
478/63	Negative	—
479/63	Positive	yes
481/63	Negative	—
482/63	Negative	—

Table V

Growth of different viruses in the different cell strains

Cell strains	adenovirus types								coxsackievirus types			
	1	2	3	5	7	8	11	14	A2	A4	A6	B3
I/1/a	+++	+++	+++	+++	+++	+—+	———	+ ?—	———	———	———	.
VIII/1/a	+++	+++	+++	+++	+++	———	———	+—+	———	—+—	———	.
II/1/a	+++	+++	+++	+++	+++	——+	——+	+++	———	—++	———	.
III/1	+++	+++	+——	+++	+——	.	.	———	.	.	.	+++
XIII/11	+++	+++	+——	+++	———	———	———	———	+——	———	———	.
XIII/12	+++	+++	+——	+++	———	———	———	———	———	———	———	.
XI/1/a	+++	+++	———	+++	———	———	———	———	———	———	———	.
primary monkey-kidn y	+++	+++	+++	+++	+——	.	.	———	.	.	.	.

Cell strains	echovirus types							parainfluenza virus types			vaccinia	polyoma virus
	3	6	7	11	12	13	19	1	2	3		
I/1/a	.	.	.	.	.	.	.	.	.	.	.	———
VIII/1/a	.	.	.	.	.	.	.	.	.	.	.	———
II/1/a	———	———	.	.	.	.	.	+—+	+++	+++	.	———
III/1	+++	+++	+++	+++	+++	+++	+++	.	.	+++	+++	———
XIII/11	.	.	.	.	.	.	.	.	.	.	.	———
XIII/12	+++	+++	+++	+++	+++	+++	+++	.	.	++	.	———
XI/1/a	.	.	.	.	.	.	.	.	.	.	.	———
primary monkey-kidney	.	.	.	.	.	.	.	.	.	.	.	———

+ = CP effect in one passage; — = No CP effect in indicated passage; . = Not tested

None of the cell strains examined supported the growth of polyoma virus.

Discussion

Both the high incidence of "spontaneous" monkey viruses and the high costs render the use of PMK cultures somewhat unfavourable for routine work. This is one of the reasons of the numerous attempts at replacing PMK by different cell strains. The sensitivity of the different cell strains to the different viruses is, however, quite variable [9, 10, 11, 12]. Studies on the cell strains developed in this laboratory were performed in order to obtain information concerning their virus sensitivities.

In agreement with other authors [10] we found the different MCS to support the growth of wild poliovirus approximately as well as PMK did, while the attenuated strains multiplied considerably better in PMK than in any of the MCS.

All faecal samples which yielded cytopathogenic agents in PMK were positive also in all MCS. Out of those negative in PMK some were found to contain cytopathogenic agents when inoculated to one or the other strain of MCS. Thus for diagnostic work the latter appeared to be more advantageous than PMK.

The virus spectrum of the individual strains of MCS differed from that of PMK, therefore the former may be used for the propagation of viruses non-infectious to PMK.

Cell strains I/1/a, II/1/a and VIII/1/a obtained from "pooled monkey-kidney" [1] primary cultures exhibited virus sensitivities different from those of the other MCS (See Table V). The former strains were found to support the growth of several adenoviruses while of none of the echoviruses. This phenomenon suggested the possibility of cell contamination of this group of MCS. Examination of such a possibility is in progress.

The sensitivity of MCS to different viruses was in general very much like that of PMK, except for strains I/1/a, II/1/a and VIII/1/a. The relatively low titres obtained in MCS with attenuated polioviruses might be brought in analogy with the known MS marker [10]. The MCS were found to be satisfactory for routine diagnostic work. Strain III/1 exhibited particularly favourable characters in view of its long survival without a change of medium and its clear-cut cytopathological reactions to virus infection. The sensitivity and virus-spectrum of all continuously maintained cell strains should, however, be tested regularly in order to reveal eventual changes brought about by prolonged cultivation *in vitro*.

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THE EPIDEMIOLOGICAL SITUATION IN HUNGARY IN INTERNATIONAL COMPARISON

By

KATALIN SOLT

State Institute of Hygiene (Director: T. BAKÁCS), *Budapest*

(Received June 1, 1964)

Summary. The morbidity rates, in certain cases also the case fatalities and mortality rates, of nine acute infectious diseases for certain European countries have been studied.

The 10 to 100fold divergencies in the registered measles morbidity result from different legal requirements for notification and from differences in the discipline of notification. The cyclic fluctuations are not simultaneous, even in neighbouring countries.

Among the countries under study, Hungary and England and Wales report the highest levels of dysentery morbidity. A comparative analysis of the morbidity, case fatality and mortality data has shown that in the countries reporting an extremely low incidence of dysentery the actual numbers of cases may amount to 50 to 100 times the reported numbers.

The incidence of infectious hepatitis is on the rise in every country except Denmark and Finland. In the latter countries the incidence shows a gradual decrease supposedly due to the immunity acquired by the population during the preceding epidemic years. In certain countries the actual incidence may be 20 to 80 fold the reported number.

The substantial decrease in diphtheria, whooping cough, tetanus and poliomyelitis in Hungary results from age-fixed compulsory vaccinations. The morbidity rate of diphtheria dropped in the period 1950—1960 to 1/16th of its initial value. It is now lower than elsewhere in Central Europe. The cyclic fluctuations of whooping cough incidence did not cease, but the morbidity rate fell to a level lower than in any earlier period. As a result of systematic vaccination with the live poliovirus vaccine the number of reported cases of poliomyelitis were, 7, 1 and 4 in the years 1961, 1962 and 1963, respectively. The incidence of tetanus fell in general to 1/3rd, for the completely immunized age groups (from 1 to 19 years of age) to 1/10th, of the initial values.

In the early 'thirties Hungary's typhoid morbidity rate was the highest in Europe. During the late 'thirties mass vaccinations and other control measures resulted in a considerable decrease. After 1945 the social-economical changes and the public health measures reduced the morbidity to a level about 5 per 100,000 population. This rate is lower than the corresponding rates for the neighbouring countries. The incidence of paratyphoid fever is relatively low in Hungary. The morbidity rate expressing the incidence of typhoid and paratyphoid is lower in Hungary than in Finland. The Hungarian rate is as high as the Swedish rate.

During the last decade, Hungary has made a considerable progress in the control of infectious diseases. The incidence of diseases preventable by vaccination has significantly dropped. In the present work an attempt was made to compare the progress in the control of communicable diseases in Hungary with the simultaneous progress in other European countries.

The epidemiological data of different countries are hardly comparable. The WHO Epidemiological and Vital Statistics Report emphasizes that for many areas of the world the numbers of notified cases do not show the real incidence of the diseases, as they only represent a variable proportion of the actual number of cases. Completeness of notification depends mainly and in varying degree on:

(1) the cultural and economical conditions of the country and the attitude of the inhabitants towards the disease *(e.g.* transfer of patients to isolation hospitals and terminal disinfection which follows notification);

(2) the legal requirements of notification.

(3) the number and the distribution of physicians and health services and the availability of diagnostic facilities;

(4) the degree of co-operation between private physicians and health authorities.

It is therefore clear that in addition to simple comparison further circumstances are to be considered before drawing conclusions.

Materials and methods

The data for the period 1950—1960 of nine acute infectious diseases were studied comparatively. The data were obtained partly from the Epidemiological and Vital Statistics Report of the WHO, partly from the statistical reports of individual countries [1—19]. As Hungarian data those of the State Institute of Hygiene, Budapest, are presented.

The nine diseases under discussion are measles, dysentery and hepatitis — each lacking adequate specific preventive measures —, and diphtheria, whooping cough, poliomyelitis, tetanus and typhoid and paratyphoid fevers, *i.e.* diseases efficiently controlled by vaccination in Hungary.

Measles

The approximately 100 per cent contagiosity of measles indicates that practically every child contracts the disease. In Fig. 1 measles morbidity for five countries are presented. The data are highly divergent. In Denmark morbidity rate fluctuates between 500 and 1500 per 100,000 population, *i.e.* it is about 15fold of that in France, where it fluctuates between 27 and 97 per 100,000 population. It should be added that during the period under study the birth rate for France significantly exceeded that for Denmark.

On the basis of the birth rates and the reported cases of measles for the period 1953—1957 KOSTRZEWSKI [20] estimates the number of notified cases in England and in Poland to 50 per cent and 12 per cent, respectively, of the actual case incidence. On the same principle we estimate the respective proportions at 25 per cent for Hungary and to 2—3 percent for France.

Measles morbidity showed neither increase nor decrease during the period under study (Fig. 1). Cyclic fluctuations occur in two- or three-year periods, but show no simultaneity even in neighbouring countries *(e.g.* in the three Scandinavian countries). The amplitude of the fluctuation is also variable. It is only twofold in Hungary and threefold in Denmark, whereas nearly twentyfold in Sweden.

In Hungary out of the notifiable acute infectious diseases measles is one of those causing the most fatal cases. According to the WHO Annuals 1954—1959, among the 16—20 European countries furnishing epidemiological

data Portugal was the only one that consistently reported measles mortality data higher than Hungary.

In the last three years of our study (1958—1960) measles mortality in Hungary amounted to 2.5, 1.1 and 1.7, respectively per 100,000 population. For the same years measles mortality for infants under one year of age amounted

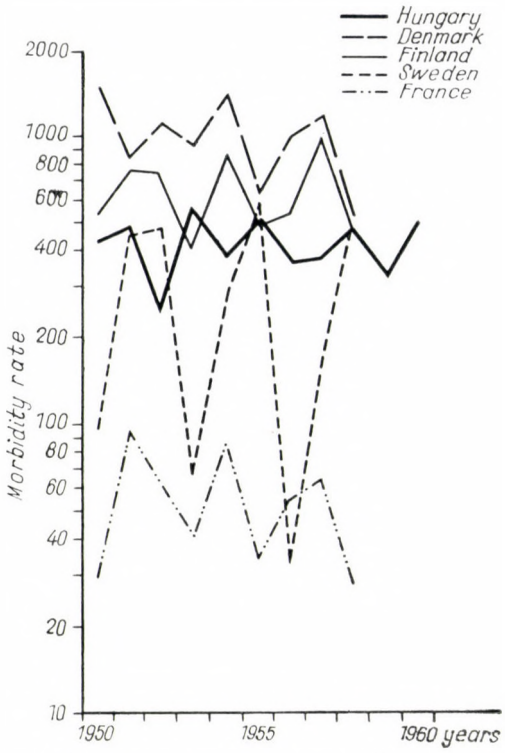

Fig. 1. Measles. Reported cases per 100,000 population

to 74.2, 29.7 and 50.3, respectively. Thus measles must not be neglected as a factor of infant mortality. For this reason Hungarian authorities expend considerable sums to protect contacts under 3 years of age with gamma globulin (0.4 ml per kg body weight). Furthermore, the health authorities are highly aware of the results of the experimental vaccination against measles.

Dysentery

In recent years, dysentery has been a serious problem in several European countries while hardly any cases have been reported from other countries.

Fig. 2 shows dysentery morbidity for eight countries. It is seen that Hungary reported the highest morbidity rate, but the figures for England

and Wales are hardly lower. In the last 3—5 years Czechoslovakia and the German Democratic Republic, too, reported high rates. Medium morbidity is shown by the German Federal Republic while only few cases of dysentery were reported from Finland, Italy and Portugal. The morbidity rates reported

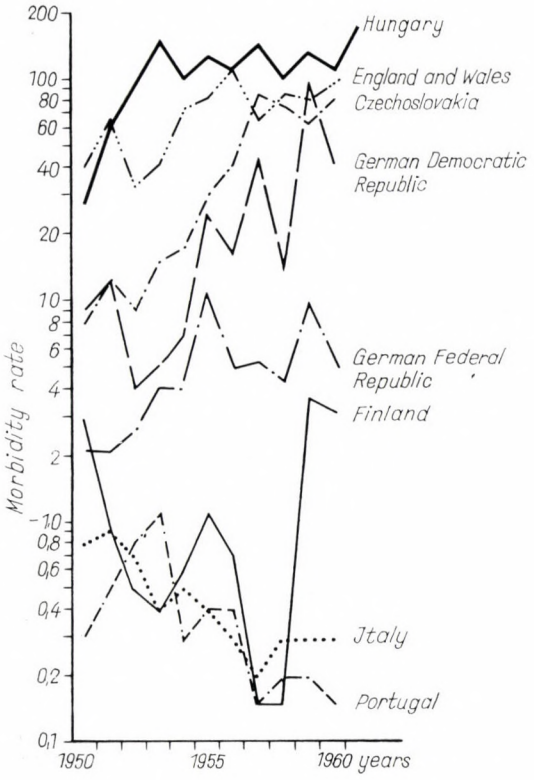

Fig. 2. Dysentery. Reported cases per 100,000 population

by Portugal and Italy are only 1/300th and 1/200th of those registered in Hungary and England and Wales, respectively.

In Table I the case fatalities are presented for the two countries reporting the highest, and the two reporting the lowest morbidity rates. In Hungary case fatality dropped from 6.9 per cent to 0.2 per cent during the period 1950—1960. In England and Wales case fatality was already low in 1950; nevertheless, it dropped to one quarter of the initial figure. In contrast, in Italy and Portugal case fatality fluctuated between 27.2 and 47.6 per cent and 12.1 and 71.4 per cent, respectively.

To obtain further data for comparison, the dysentery mortality rates (Table II) for the same countries were also studied. The highest mortality

Table I

Dysentery. Case fatality for several European countries

Country	1950	1951	1952	1953	1954	1955	1956	1957	1958	1959	1960
Hungary	6.9	6.4	3.0	2.7	1.3	1.3	0.8	0.5	0.6	0.4	0.2
England and Wales	0.3	0.2	0.2	0.2	0.1	0.1	0.06	0.07	0.08	0.09	0.08
Portugal	19.3	15.9	40.0	30.0	52.0	54.8	12.1	28.5	66.6	68.4	71.4
Italy	27.7	27.2	28.0	39.6	28.2	29.6	40.4	30.8	47.6	32.2	28.5

rate was shown by Hungary though in this country its value dropped to 1/15th of the 1951 level. In Italy and Portugal, on the other hand, mortality is low, hardly different from that for England and Wales.

Table II

Dysentery. Death rates per 100,000 population for several European countries

Country	1950	1951	1952	1953	1954	1955	1956	1957	1958	1959	1960
Hungary	1.9	4.4	2.8	4.1	1.3	1.6	0.9	0.7	0.6	0.5	0.3
England and Wales	0.1	0.2	0.08	0.08	0.09	0.09	0.1	0.0	0.1	0.1	0.06
Portugal	0.07	0.08	0.3	0.3	0.1	0.2	0.1	0.0	0.2	0.2	0.05
Italy	0.2	0.2	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.05

Dysentery being an enteric infectious disease its spreading is influenced by social-economical cultural and communal-hygienic factors. Climatic factors may also play a role *e.g.* in the low incidence of dysentery in Northern Europe.

The incidence of dysentery is rising in six out of the eight countries shown in Table II while in Italy and Portugal the number of notified cases shows a decline. This trend cannot be explained by the above discussed facts.

It can hardly be believed that in England and Wales the economical cultural and communal-hygienic conditions might be responsible for a morbidity rate 200 times higher than those for Italy and Portugal.

To find an explanation we compared the trends of the case fatality and mortality data. In the countries reporting high morbidities the case fatality was gradually decreasing while in those reporting low morbidity data it fluctuated at high levels. In the latter countries one quarter to three quarters of the notified cases were fatal. High fatality together with low mortality indicates, in general, a very severe disease. However, since the introduction of new therapeutics dysentery ceased to be a severe disease.

It can thus be concluded that in the countries reporting extremely low dysentery morbidity rates the actual number of cases may be 50—100fold the number of notified cases.

In Hungary dysentery has attracted special interest since the early fifties. As a result, the registered morbidity attained, within three years a level five times higher than the 1950 one. This virtual increase was due to the following factors.

(1) Introduction of compulsory free-of-charge laboratory examination of the dysentery patients and suspect cases.

(2) Use of the sodium-desoxycholate medium, a sensitive procedure for detecting Shigella infections.

(3) Organization of postgraduate courses on diarrhoeas, and especially on dysentery.

(4) New categories of the population were involved in the social insurance.

(5) Organization of the network of county epidemiologists.

As a result of these measures mild cases of dysentery were also seen by the physician thus were notified. (It should be noted that according to DOBSZAY [21] only about 1/10th of the actual cases had been notified in Hungary before 1945.)

Thus, two factors are responsible for the decrease in the case fatality of dysentery in Hungary, namely

(a) the improvement of therapy has led to a significant decrease in mortality;

(b) mild cases are also notified.

The more than eight-fold and more than 20fold increases in the incidence in Czechoslovakia and the German Democratic Republic, respectively, may also be explained by similar factors. The public health authorities are greatly interested in dysentery in those countries, too, and the antagonism between the execution of the epidemiological measures and the existential interests of the physicians has been eliminated as a result of the large-scale social insurance. This is characteristic of the social-hygienic care also in England and Wales.

Dysentery is therefore one of the unsolved public health problems of Hungary as well as of other countries. Its control requires wide-scale measures. In spite of the high morbidity rate, the mortality due to dysentery has significantly decreased since the introduction of appropriate therapy.

Infectious hepatitis

Infectious hepatitis has been a serious epidemiological problem in the last two decades all over the world. The disease had been made notifiable in most European countries at about 1950.

In Fig. 3 the incidence of hepatitis in eight European countries is illustrated. In the early 'fifties six curves showed a steep rise due to the improving discipline of notification and diagnostic methods. Thus, the increase in incidence was mostly virtual.

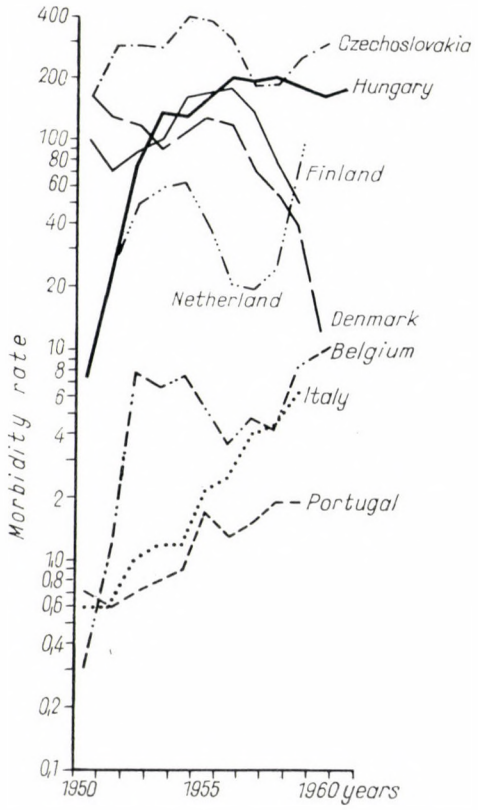

Fig. 3. Infectious hepatitis. Reported cases per 100,000 population

In two countries, Denmark and Finland, the trend was inverse, showing a gradual decrease during the late 'fifties. To find an explanation for this decrease, we analyzed in this respect the epidemiological date for the preceding decades. Denmark had experienced a country-wide hepatitis epidemic with extremely high morbidity rates during the 'thirties and World War II. In Finland the incidence was high during World War II, when the country was under German occupation. RAŠKA [23] MILOJČIČ [24] and MCCOLLUM [25] assume a cyclic fluctuation in hepatitis morbidity. The gradually decreasing trend shown by Denmark and Finland suggests that the population of these

Table III

Infectious hepatitis. Case fatality for several European countries

Country	1950	1951	1952	1953	1954	1955	1956	1957	1958	1959	1960
Hungary	1.4	0.6	1.0	1.3	1.3	1.0	0.9	0.7	0.7	0.5	0.7
Denmark	0.3	0.4	0.5	0.6	0.7	0.5	0.4	0.5	0.6	0.4	0.7
Finland	0.4	0.7	0.9	0.5	0.2	0.3	0.3	0.3	0.4	0.3	0.3
Czechoslovakia	0.6	0.4	0.4	0.4	0.3	0.3	0.4	0.5	0.4	0.4	0.3
Netherlands	...	2.0	0.9	1.2	0.8	1.4	2.0	1.8	1.1	0.4	0.4
Switzerland	5.3	2.6	3.6	2.3	1.7	2.3	4.2	6.2	8.6	5.7	6.8
Italy	...	13.6	14.7	20.1	8.1	3.0	1.4	9.0	7.8	5.9	...
Portugal	8.9	27.2	20.6	7.4	10.8	35.9	24.1	25.0	24.5	23.0	23.0
Belgium	...	...	...	...	6.4	11.2	14.3	14.1	15.4	8.8	7.5

countries had acquired immunity during the epidemic years and at present a cycle is approaching its bottom level.

The peculiar morbidity curve of the Netherlands may show another type of cyclic fluctuation.

The morbidity levels being highly variable, it seemed to be of interest to investigate the case-fatality and morbidity data to obtain a firm basis for comparison.

Table IV

Infectious hepatitis. Death rates per 100,000 population for several European countries

Country	1950	1951	1952	1953	1954	1955	1956	1957	1958	1959	1960
Hungary	0.1	0.1	0.7	1.8	1.7	1.6	1.7	1.4	1.3	0.9	1.1
Denmark	0.5	0.6	0.5	0.7	0.5	0.4	0.4	0.4	0.3	0.2	0.2
Finland	0.4	0.5	0.8	0.5	0.3	0.5	0.4	0.3	0.3	0.3	0.1
Czechoslovakia	1.0	1.0	1.2	1.1	1.1	1.1	1.1	0.9	0.8	0.9	0.8
Netherlands	0.4	0.5	0.4	0.5	0.5	0.5	0.4	0.4	0.3	0.4	0.5
Switzerland	0.8	0.6	1.1	1.3	1.3	1.6	1.2	1.3	1.6	1.3	1.3
Italy	...	0.08	0.2	0.2	0.2	0.3	0.4	0.4	0.4	0.4	...
Portugal	0.06	0.1	0.1	0.06	0.09	0.6	0.3	0.4	0.5	0.5	0.5
Belgium	...	...	...	...	0.5	0.6	0.5	0.7	0.6	0.7	0.7

According to Table III, case fatality fluctuated around or below 1 per cent in the countries reporting high morbidity rates. In Switzerland showing medium morbidities, from 15 to 70 per 100,000 population, case fatality ranged from 1.7 to 8.6 per cent. In the countries reporting low morbidities

case fatality was high (Italy, 1.4 to 20.1 per cent; Belgium, 6.4 to 15.4 per cent; Portugal, 7.4 to 35.9 per cent).

Table IV shows, on the other hand, that the mortality rate was at the same level in Hungary as in Switzerland. In Italy, Belgium and Portugal the mortality rate is equal to, or lower than, in Denmark, Czechoslovakia and Finland, in spite of the high differences in case fatality.

One may conclude that hepatitis resembles dysentery in that in the countries reporting low morbidities the actual incidence may exceed 20 to 80 times the number of notified cases.

The high incidence reported by Hungary appears to be real, as suggested also by McCollum [25]. Recently the morbidity level has been unchanged for years. It is believed that the systematic administration of gamma globulin for the hepatitis contacts (0.02 ml per kg body weight) of a 10 per cent solution prevents further increases in the incidence. On the other hand, hepatitis cannot be controlled before its aetiology has been revealed and mass vaccinations have been organized.

Diphtheria

The incidence of diphtheria has gradually been decreasing in most European countries since the end of World War II. The decline has been the greatest in Finland (200fold). In England and Wales the incidence dropped to 1/10th of the initial level, which was the lowest morbidity rate in the countries under study in 1950. In Hungary the reduction was 16fold during the period from 1950 to 1960 and since 1960 the decrease has accelerated. In Czechoslovakia the morbidity rate for 1950 and 1951 was about the same as in Hungary, but since then the decrease has been slower.

In 1950, Austria, the German Democratic Republic and Poland showed the highest mortality rates. In the first two countries the incidence has dropped to 1/5th of the 1950 level during the last 11 years.

The situation in Poland deserves special interest. In this country the incidence of diphtheria was increasing until 1955. According to Kostrzewski [20], Poland experienced such a great diphtheria epidemic between 1947 and 1955 as never during the preceding 70 years. This author attributed the epidemic to the following factors.

(1) During, and immediately after, World War II vaccinations were neglected; (2) the discipline of notification had improved; (3) birth rate had increased; (4) a significant part of the population had assembled in newly constructed large towns; and (5) the number of children nursed in welfare institutions had suddenly grown.

By 1960, as a result of systematical vaccinations, the incidence of diphtheria dropped to 1/15th of the 1955 level.

Out of the nine countries under study, Italy showed no remarkable change in morbidity while in Belgium some increase appeared during the late 'fifties.

In Hungary, according to the data of the State Institute of Hygiene, the yearly incidence of diphtheria fluctuated between 14,000 and 19,000

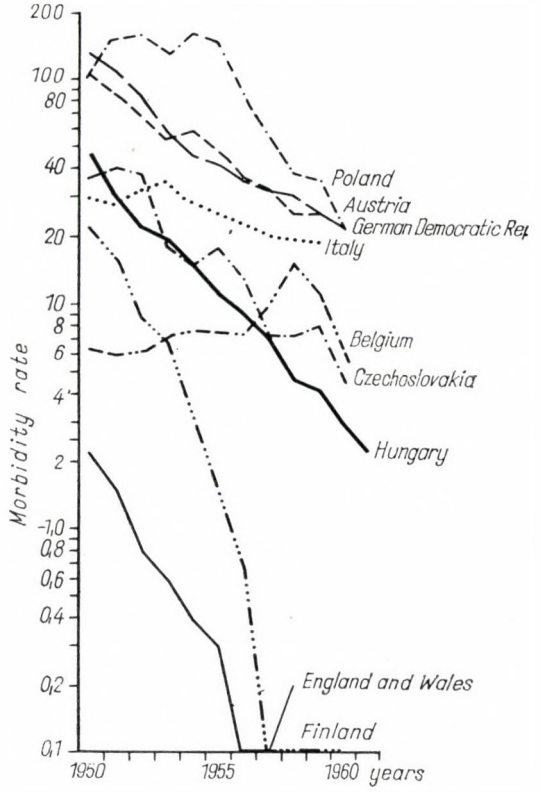

Fig. 4. Diphtheria. Reported cases per 100,000 population

(154.2 and 221.7 per 100,000 population) during the period 1931 to 1934. At the same time, 1000—1500 children died of diphtheria (9.4—17.3 per 100,000 population) every year. Experimental vaccination was initiated at the middle of the 'thirties, and vaccination was made compulsory in 1938 (vaccination at 2 years, and revaccination at 6 years of age). As a result, morbidity showed a definite decline. Hungary was the second country (after Poland) to introduce compulsory vaccination, in 1938.

Since 1947 the vaccination schedule has been changed several times. In the period 1953—1960 children received basal immunization (two injections

at an interval of four weeks) in the second half year of life; the first revaccination took place 1 year later and the second at 6 years of age. Since 1953 adsorbed DPT vaccine has been employed.

The examples of Finland and England and Wales have shown that systematic vaccination may be highly effective in the control of diphtheria within a short period of time. Finland's diphtheria morbidity highly exceeded the Hungarian level during World War II (in 1943 it was roughly 400, in 1944 450 per 100,000 population, [27]) but as shown in Fig. 4 it has fluctuated around 0.1 per 100,000 population since 1957. England and Wales reported 120 cases per 100,000 population for 1941, *i.e.* a rate highly exceeding the simultaneous Hungarian rate. Since 1956, however, the morbidity rate for England and Wales has been moving between 0.1 and 0.2 per 100,000 population.

Further accurate execution of the vaccination programme is expected to lead to similar results in Hungary.

Whooping cough

Whooping cough belongs to the highly contagious droplet infections. A significant proportion of the non-immunized children population contracts the disease. In this case the registered morbidity depends on two factors, namely the completeness of notifications and the extent of vaccinations.

Fig. 5 shows the whooping cough morbidity data for 10 countries. The highest rates are shown by the Scandinavian countries, the lowest by Portugal and France. The divergence is approximately 100fold.

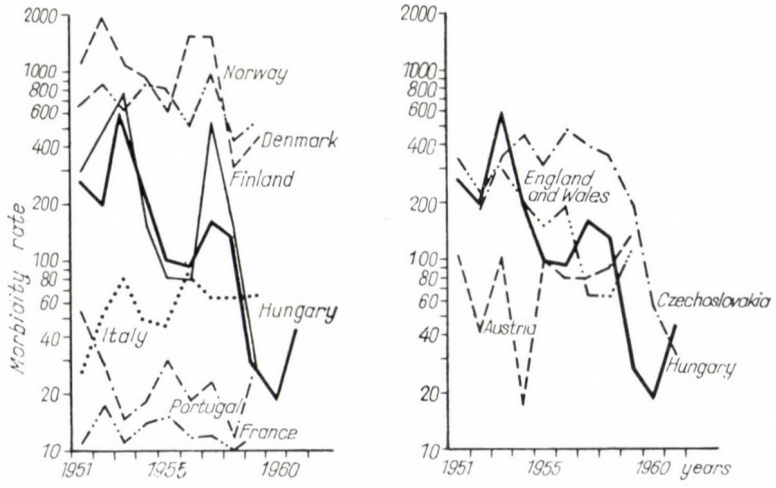

Fig. 5. Whooping cough. Reported cases per 100,000 population

Most of the 10 countries show irregular, in general 3- to 5-year cycles. The trend is definitely decreasing in Hungary, Finland, Czechoslovakia, Denmark and England and Wales.

According to the WHO statistics and the literature, Hungary was the first (in 1953) to introduce age-fixed compulsory vaccination against whooping cough (the vaccination schedule see under Diphtheria). Cyclic fluctuation could not be eliminated by vaccination, but the incidence fell to a significantly lower level. The clinical course of the disease has become definitely milder. Mortality began to decline before the introduction of compulsory vaccination, owing to the extensive antibiotic therapy [28]. The Central Bureau of Statistics reported only 15 and 7 fatal cases for 1959 and 1960, respectively.

Fig. 5 shows the striking resemblance between the morbidity curves for Finland and Hungary. In 1957 (as well as in 1961—1962), however, the peaks of the Hungarian curve were considerably lower than those of the curve for Finland. The difference might have resulted from the difference in vaccination. According to the WHO Annuals the rate of vaccinations was double the respective Finnish rate during the period 1954—1958.

Czechoslovakia introduced vaccination against whooping cough in 1957—1958. The morbidity curve has shown a significant decline since 1959. Nevertheless, the time elapsed since the start of compulsory vaccination is too short for any definite statements.

It can be concluded that vaccination against whooping cough has been successful in Hungary. Since 1960—1962 vaccination (in the form of DPT) is started at three months of age and since 1963 basal immunization consists of three injections. It is hoped that these modifications will further decrease the incidence and eliminate mortality of whooping cough among infants.

Poliomyelitis

Considering that the introduction of compulsory vaccination in the late 'fifties has completely changed the epidemiological situation of poliomyelitis, we shall include the 1961 and 1962 data into analysis.

Fig. 6 illustrates the morbidity curves for six countries. In these countries poliomyelitis epidemics occurred in every third to sixth year. The Hungarian curve showed a peak in every fifth or sixth year up to 1952. In the period 1954—1959 four epidemics were experienced [29].

Before the summer of 1957, the control of poliomyelitis consisted of measures usually applied against enteric infections. Hungary experienced her greatest poliomyelitis epidemic in 1957, with 2334 cases (23.8 per 100,000 population). Vaccination was started at the peak of that epidemic. In the same year 1,200,000 children, *i.e.* 80.4 per cent of the population under six years

of age, were immunized with Salk vaccine, free of charge. By the end of 1958, 2,000,000 subjects, *i.e.* 70 per cent of the population under 18 years of age had been immunized, each with three doses of the Salk vaccine.

The number of cases prevented by the vaccination carried out in face of the 1957 epidemic was estimated to 500 by Petrilla [31] and to 2000 by Bakács [32].

The year 1958 was outstandingly favourable for Hungary as regards poliomyelitis. In that year as few cases occurred as in none of the preceding

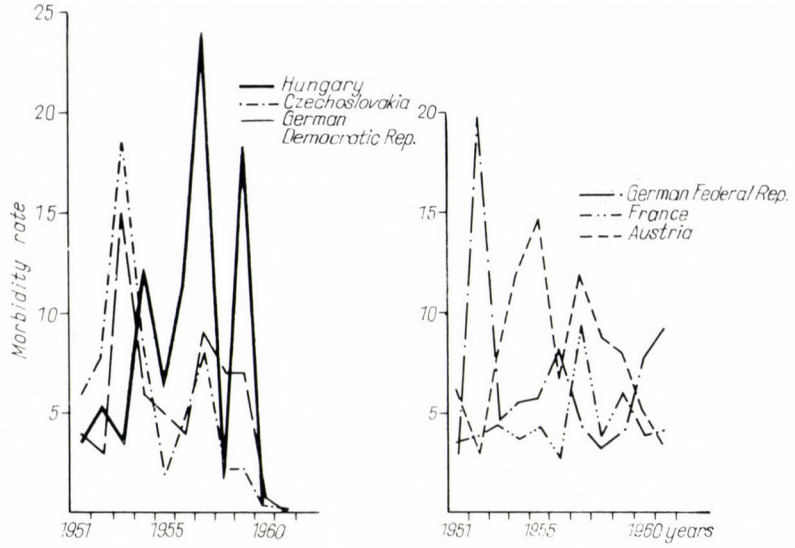

Fig. 6. Poliomyelitis. Reported cases per 100,000 population

20 years. The low incidence was attributable to three factors *viz.* (a) the immunity acquired during the preceding epidemic year; (b) the interfering activity of the simultaneous Bornholm disease epidemic; and (c) the vaccination [33].

In 1959, however, another severe epidemic developed, in spite of the mass vaccinations; 1830 cases were reported (18.3 per 100,000 population). Only the 1957 epidemic was larger in Hungary than this one. Rudnai and Barsy [34] estimate to 1132 the number of cases among children under 14 years of age which were prevented by the immunization carried out during the preceding two years with the Salk vaccine. In general, the protection provided by the vaccination was estimated to 60—65 per cent.

In December, 1959, and January and February 1960, approximately 2,500,000 children received the live oral vaccine prepared from Sabin's vaccine strains in the USSR. Thus Hungary was the first country all over the world

where a proportion as large as 92.4 per cent of the population under 15 years of age had received live poliovirus vaccine [30]. Since that time, vaccinations and revaccinations have been carried out according to a well-defined programme. As a result of these the yearly incidence of poliomyelitis fell to 38, 7, 1 and 4 in the four years 1960—1963. In some of these cases the clinical diagnosis of poliomyelitis could not be confirmed by virological tests.

From Czechoslovakia no case of poliomyelitis has been reported since 1961. From the German Democratic Republic two cases were reported in 1961, no cases in 1962 and 1963.

Among the countries where the live vaccine has not been applied on a wide scale Austria, France and the German Federal Republic registered 238, 1151 and 4627 cases, respectively, in 1961.

Since then most of the European countries have accepted the live poliovirus vaccine, thus the eradication of the disease has begun. The realization and acceleration of this process has singificantly been supported by the Hungarian experience.

Tetanus

The relatively great number of fatal cases provides an epidemiological importance for tetanus in Hungary. In Table V only the mortality data are presented because most countries do not report tetanus morbidity. Table V shows that the tetanus morbidity rate is significantly higher in Hungary and in the countries lying south of Hungary than in the northern countries.

Table V

Tetanus. Death rates per 100,000 population for several European countries

Country	1951	1952	1953	1954	1955	1956	1957	1958	1959	1960
Hungary	2.5	2.5	1.9	1.7	1.7	1.5	1.1	1.2	1.0	0.8
Yugoslavia	2.2	2.5	2.2	1.9	1.7	1.5	1.3	3.8	1.4	...
Italy	1.4	1.5	1.4	1.2	1.1	1.2	1.1	1.0	...	...
Austria	1.3	1.3	1.2	1.0	0.9	1.0	1.0	1.0	0.9	0.6
German Federal Republic	0.7	0.5	0.4	0.4	0.4	0.4	0.4	0.3	...	...
Denmark	0.5	0.5	0.4	0.3	0.6	0.3	0.2	0.3	0.2	...
Finland	0.2	0.1	0.01	0.2	0.2	0.1	0.09	0.09	...	...
Czechoslovakia	...	...	...	...	...	0.5	0.7	0.6	0.4	0.5

The great number of deaths made the Hungarian authorities to introduce vaccination against tetanus in 1953 by incorporating tetanus toxoid

in the diphtheria pertussis vaccine, and typhoid-tetanus vaccine has been administered to children at 12 years of age and the same typhoid-tetanus vaccine has been used for large-scale vaccinations against typhoid fever.

As a result of the vaccinations, by 1960 the general incidence of tetanus and its incidence in the completely vaccinated age groups (from 1 to 19 years of age) had dropped to 1/3rd and 1/10th, respectively, of the initial levels. The decrease in mortality was threefold [35].

The 80 per cent reduction in neonatal tetanus in the period 1951—1960 was due to an increase in the percentage of institutional births (41.4 per cent in 1951, 85.0 per cent in 1960).

In addition to these factors the rise of the life standard of the agricultural workers (many of whom had walked barefoot before) and the improving medical care have contributed to the decrease in the incidence and mortality of tetanus.

To achieve further reduction, wide-scale vaccination of older agricultural workers would be advisable.

Typhoid and paratyphoid fevers

The success of the fight against typhoid fever is in close connexion with the economical, cultural and public health developments of a country [22]. For this reason we review here a longer period of time than in the case of other diseases.

In Fig. 7 the typhoid morbidity rates of six countries for the period 1926—1955 are presented. At the beginning of this period (1926—1930) Hungary was the last but one among 33 European countries as regards typhoid morbidity (according to the data by the League of Nations). The average morbidity rate was as high as 106 per 100,000 population, the average death rate 16.5 per 100,000 population *(i.e.* 10 and 20 times the death rates for Germany and Denmark, respectively [37]). During the period 1931—1935 among the 26 European countries listed in the League of Nations report, Hungary showed the highest morbidity rate (111.1 per 100,000 population). For the same period the mortality rate was 27.2 per 100,000 population.

These data truly reflect the economical, cultural and public health levels in Hungary in the early 'thirties.

From the middle of the 'thirties on as a result of wide-scale vaccinations and other epidemiological measures, the incidence of typhoid fever gradually declined until World War II. During the war, like elsewhere in Europe, the morbidity curve had another peak. According to BAKÁCS [37] the average yearly decrease was 10.3 per cent for the period 1931—1945, in contrast to the 15.8 per cent average of the years 1945—1958.

Fig. 8 shows the changes in typhoid morbidity during the period 1950—1960. In contrast to the 'thirties, in 1950 the morbidity rate for Hungary moved at the average European level and during the 'fifties it dropped to 1/3rd of the 1950 level.

During the same period the incidence of typhoid fever decreased in Czechoslovakia by 80 per cent, in the Netherlands, where it was low in 1950

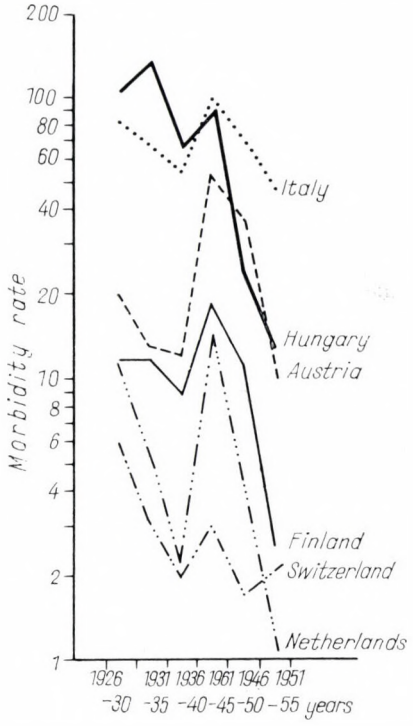

Fig. 7. Typhoid fever. Average numbers of reported cases per 100,000 population for the period 1926—1955

already by about 50 per cent. In Finland the reduction was about 75 per cent, while in Poland morbidity is still high, in spite of the 60 per cent decrease during the period 1950—1960.

In Italy and Portugal the incidence of typhoid fever showed no appreciable decline during the 'fifties. The morbidity rates for these countries are among the worst rates of Europe.

The drop of typhoid morbidity in Hungary after 1945 was supported by the social-economical changes and the rise of the life standard and the cultural level in general, and the rise of the hygienic culture, in particular. In 1930, the number of physicians per 100,000 population was 5.6, in 1950,

11.0 and in 1960, 15.7. In 1931, 25 per cent; in 1950, 47 per cent; in 1960, 85 per cent, of the population was involved in the social insurance [18].

The systematically executed hygienic-epidemiological measures including free-of-charge isolation of patients in hospitals, laboratory examinations, the search for, and severe control of carriers and the mass vaccinations were

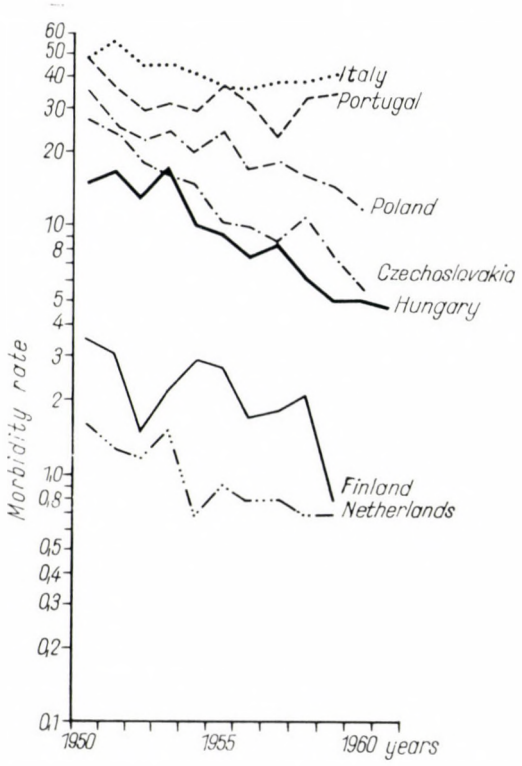

Fig. 8. Typhoid fever. Reported cases per 100,000 population

the most important factors resulting in the great reduction of the incidence of typhoid fever.

It is, however, easier to reduce an excessively high incidence to the average level than to achieve further reduction of relatively favourable morbidity rates. Since 1959, typhoid morbidity has fluctuated around 5 per 100,000 population. Any further reduction needs complex epidemiological and public health activities [38].

In several European countries the incidence of paratyphoid is higher than that of typhoid fever. Fig. 9 shows that in Hungary the incidence of paratyphoid was relatively low during the period 1926—1955.

Table VI

Typhoid and paratyphoid fevers. Death rates per 100,000 population for several European countries

Country	1956	1957	1958
Hungary	9.1	9.9	7.6
Italy	46.5	44.8	46.9
Spain	42.0	34.1	42.0
Greece	31.1	39.1	32.2
Finland	26.1	16.3	18.5
Sweden	6.6	14.0	7.6
Netherlands	2.8	3.7	3.2
England	1.3	1.0	0.8
Denmark	1.7	0.7	0.6
Norway	0.7	0.4	0.2

In several countries including France and Spain, typhoid and paratyphoid fevers are reported together. For this reason we present the WHO data on typhoid and paratyphoid morbidity for the years 1956—1958 in Table VI. It is seen that in this respect the situation is worse in Finland than in Hungary.

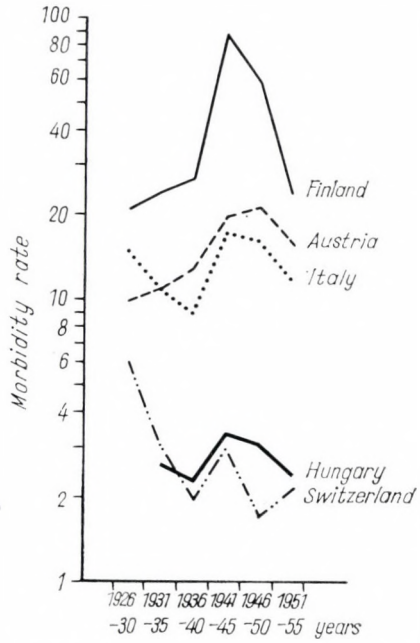

Fig. 9. Paratyphoid fever. Average numbers of reported cases per 100,000 population for the period 1926—1955

It is not more favourable even in Sweden, where, typhoid morbidity is as low as 0.1—0.3 per 100,000 population.

It should, however, be emphasized that the morbidity data for the Netherlands, Denmark, Norway, Belgium and England and Wales are significantly more favourable than those for Hungary, even in this respect.

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TRANSFORMATION OF STREPTOMYCIN MARKERS IN ROUGH STRAINS OF RHIZOBIUM LUPINI

TRANSFORMATION OF STREPTOMYCIN-DEPENDENCE

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Summary. The transformation of streptomycin-dependence in rough strains of *Rhizobium lupini* has been studied.

(i) The transformation frequency of streptomycin-dependence is very low, 0.0002 to 0.000001 per cent in cases of "heterologous" transformation, and 0.0004 to 0.024 per cent in the case of "homologous" transformation.

(ii) The markers of streptomycin-resistance and dependence can be transferred both separately and together.

(iii) The same preparation of DNA is able to transfer both a stable and an unstable dependence.

(iv) The level of streptomycin-dependence or resistance obtained by transformation is different in the different groups of transformants, one group reaching the original level of the donor, the other showing a lower level.

(v) Among the transformant colonies the large ones are resistant, the normal and small ones are either dependent or resistant.

(vi) It has been concluded that in Rhizobium the mutational sites of streptomycin-dependence and resistance are localized in one complex locus.

Genetic analysis of the difference between the single-step and multi-step type of mutation at a high level of streptomycin-resistance has been performed by transformation experiments with *Diplococcus pneumoniae* [6, 8, 11], recombination with *E. coli* [14], and transduction experiments with *S. typhi murium* [15, 16]. Genetic analysis of streptomycin-dependence has also been carried out by recombination experiments with *E. coli* K-12 [5, 10, 13], and transduction with *E. coli* [7, 9] and *S. typhi murium* [5]. As the analysis of the locus of streptomycin-dependence was made only by HASHIMOTO by means of transformation with *Diplococcus pneumoniae* [6], our aim was to study (1) whether it was possible to transform with the DNA of single-step, highly resistant streptomycin-dependent mutants of the rough, streptomycin-sensitive strains of *Rhizobium lupini* [4] and (2) whether or not the working hypothesis of DEMEREC *et al* [5], NEWCOMBE and NYHOLM [10], *i.e.* that resistance to high concentrations of streptomycin, and dependence on streptomycin are controlled by alleles of the same complex gene locus, or by two loci in very close linkage, would be valid for Rhizobium?

*Deceased in 1960.

Materials and methods

Bacterial strains. Rough strains of *Rhizobium lupini* were used. The strains H-13-1 and H-13-3 were streptomycin-sensitive [4], the others trains were streptomycin-dependent mutants of strains H-13-1 and H-13-3. The dependent strains were obtained by single-step selection of spontaneous mutants from sensitive strains or by selection of dependent transformants in experiments of transformation of streptomycin-dependence. During the selection of spontaneous mutants, we obtained one dependent colony, selected with 1000μg/ml of streptomycin from 1×10^{11} cells (strain H-13-1) and 5×10^{9} cells (strain H-13-3), respectively. The selected strains grew slowly on media containing 100 or 300 μg/ml streptomycin. The streptomycin requirement of the dependent strains obtained by transformation of streptomycin-dependence was only 300 μg/ml. The response to streptomycin of the strains used is shown in Table I.

Media. The nutrient medium contained (per liter) K_2HPO_4, 3.6 g; KH_2PO_4, 0.4 g; $MgSO_4 \cdot 7H_2O$, 0.05 g; NaCl, 0.5 g; ferric ammonium citrate, 4.0 mg; agar, 20 g. The pH was adjusted, to 7.0 — 7.2. To the synthetic medium were added glucose to a final concentration of 5 g, casein hydrolysate 1 g, and yeast extract 1 g, for 1000 ml [2].

Preparation of DNA. The bacteria were centrifuged at the end exponential growth, resuspended in 0.1 *M* NaCl sodium citrate, and lysed by the addition of 0.2 volume of 5 per cent deoxycholate for 10—15 min at 55°C. The material was deproteinized 5—7 times by the SEVAG method with chloroform and purified by repeated precipitation in 3 volumes or 1 volume of alcohol, respectively. The DNA thus obtained was stored by the method of ZAMENHOF in 0.14 *M* NaCl and 0.015 *M* sodium citrate at pH 7.4, at 4°C. The DNA content of the preparations was estimated according to DISCHE [2]. The transforming extract contained no viable bacteria.

Deoxyribonuclease (DN-ase). The DN-ase used was prepared in the Institute of Medical Chemistry, University Medical School, Budapest.

Transformation technique. A 20 hrs old culture (10^8 bacteria per ml) was diluted to a final concentration of 10^8 bacteria/ml into 10—15 ml fresh supplemented medium, containing 5—19 μg/ml of the transforming DNA. The transformation reaction was stopped, if necessary, by the addition of DN-ase at a final concentration of 2.5 μg/ml. In other cases the transformation reaction lasted 8, 24 or 48 hours without addition of streptomycin to the medium. Thereafter the transformed population was plated on the selective medium, containing 30, 100, 300 or 1000 μg/ml streptomycin, and incubated for 48 to 72 hours at 30°C. As controls, cultures of cells grown under identical conditions but without transforming DNA were plated on the selective media to verify that the number of spontaneous mutations bearing the marker under study was insignificant. The frequency of transformation was calculated in per cent of the total number of cells, estimated on medium lacking streptomycin.

The colonies grown on the selective medium were tested for character by suspending in 0.5 ml of saline some colonies picked up at random, and streaking a loopful on media containing 0, 30, 50, 100, 300 or 1000 μg/ml streptomycin. After 48 to 72 hours incubation at 30°C growth was scored from — to +++++, or the colonies were counted.

Results

Genetic analysis of streptomycin-dependent donors (spontaneous mutants or transformants) have revealed the following. Table II gives the data of experiments performed to study whether streptomycin-dependence could be transferred by transformation, and to determine the most suitable conditions for detecting the streptomycin-dependent transformants. The recipient bacteria were sensitive to streptomycin, transformation was carried out without streptomycin (Tables I, II).

In the experiment with the DNA of the streptomycin-dependent strain 1-str-d $_{(1000)}$ (Table II, exp. I/74) seven normal-sized colonies and one small colony among ten, which appeared on selective medium, were streptomycin-independent and had a high level of resistance. Testing of two very small

Table I

Growth of the strains of Rhizobium lupini on agar containing various concentrations of streptomycin

STRAINS	STREPTOMYCIN CONCENTRATIONS (μg/ml)								
	0	0.4	1	2	4	30	100	300	1000
Donors									
(H—13)-1-str-$d_{(1000)}$	—	—	—	—	—	—	++	+++	+++++
(H—13)-3-str-$d_{(1000)}$	—	—	—	—	—	—	+++	++++	+++++
(H—13)-3-str-d-$15_{(1000)}$	—	—	—	—	—	—	+++	++++	+++++
(H—13)-3-str-$td_{(300)}$	—	—	—	—	—	—	+++	++++	++
(H—13)-3-str-d-$td_{\mu(300)}$	—	—	—	—	—	—	++	+++++	+
Recipient									
(H—13)-3-str-s	+++++	+++++	++++	++	—	—	—	—	—

str-s: streptomycin-sensitive strain, str-d: spontaneous, single-step, high-level streptomycin-dependent mutant, str-td: streptomycin-dependent strain obtained by transformation. The index shows the concentration of streptomycin (μg/ml) required for optimal growth. In the text the sign of the strains will be used in abbreviated form, without the parts in parentheses.
—absence of growth; + to +++++ rate of growth on media containing different concentrations of streptomycin after 48hr incubation at 30°C.

Table II

Transformation of streptomycin-dependence with different DNA-s

Number of experiment	Donors of DNA	DNA in μg/ml	Duration of the transformation reaction, hrs.	Initial number of recipient cells/ml	Transformation volume ml	Initial total number of cells	Distribution of transformants of the total transformation volume on plates, containing μg/ml streptomycin				Frequency of transformation
							30	100	300	1000	
I/74	1-str-d$_{(1000)}$	25	48	1.0×10^7 1.0×10^6	2 2	2.0×10^7 2.0×10^6	— —	2 2	— —	4 2	0.00003 0.0002
II/22	1-str-d$_{(1000)}$	12	24 48	9.3×10^6	10	9.3×10^7	0 2	— —	1 1	0 0	0.000001 0.000003
II/26	3-str-td$_{(3000)}$	12	24 48	2.1×10^5	10	2.1×10^6	60 35	— —	31 6	— —	0.0091 0.0041
II/5	3-str-td$_{\mu(300)}$*	12	8 24 48	1.0×10^6	2	2.0×10^6	— — —	0 48 587	— — —	0 6 700	0.000000 0.0027 0.0643
II/53	3-str-d$_{(1000)}$		24 48	2.4×10^5	15	3.6×10^6	3 211	2 133	1 25	0 0	0.0004 0.024

Recipient of DNA: strain 3-str-s.

* These data were obtained in 0.1 ml of the total volume of transformation.

Assay of transformants and of the total number of cells was carried out in the following way. After the addition of DNA to the recipient culture and its incubation for the indicated time, the population was centrifuged and resuspended in saline (in one tenth of the original volume if a large transformation volume 10 or 15 ml was used). An aliquot of 0.2 ml was used to make an appropriate dilution for the determination of the total number of cells in nonselective media. To assay the transformants, several samples, each with 0.1 ml of the remaining volume, were plated on selective media containing different concentrations of streptomycin. If the volume of the transformation medium was 2 ml, the procedure was the same but without concentration of the cells.

colonies appearing on plates containing 1000 μg/ml streptomycin showed, if isolated, transferred into liquid medium and then plated on media lacking or containing streptomycin, no colonies formed on the first medium after 24 to 48 hours, a phenomenon indicative of streptomycin-dependence. Growth was abundant on 100 μg/ml and weak on 1000 μg/ml. After 48 hrs colonies appeared on the plates lacking streptomycin. If, after the appearence of these colonies, the agar layer was cut out and placed on a layer containing streptomycin (in an amount sufficient to ensure a final concentration of 100 μg/ml of streptomycin in each layer), and re-incubated for 48 hrs, new colonies arose in numbers corresponding to about 5 per cent of the originally counted population. These new colonies were analyzed again on plates lacking or containing streptomycin. The re-isolated colonies behaved exactly like the original colonies,

being dependent during 24 to 48 hrs and growing after 48 more hrs on the medium lacking streptomycin. We have termed this phenomenon "unstable" dependence.

In experiment II/22, of the four resistant transformants one appeared on a plate containing 300 μg/ml streptomycin and proved to be stable streptomycin-dependent, having a lower level of resistance as the donor and growing optimally at a concentration of 300 μg/ml of streptomycin, meanwhile on 100 and 500 μg/ml streptomycin growth was slow.

Next, transformation of streptomycin-dependence with DNA of stable and unstable streptomycin-dependent transformants was studied. Table II shows that in experiment II/26, where transformation was carried out with the DNA of the streptomycin-dependent transformant strain 3-str-td$_{(300)}$ the frequency of transformation increased by several orders of magnitude in agreement with our previous data [3] and those of SCHAEFFER [12]. The colonies, appearing at streptomycin concentrations of 30 and 300 μg/ml were further analyzed according to their size on different media with or without streptomycin (Table III).

The data obtained indicate the following. (1) During the 24 and 48 hrs transformation reaction part of the colonies was resistant, another part was dependent, and part of them sensitive (persistent). (2) The dependent transformants were always found among small and normal-sized colonies. The big colonies were all resistant (streptomycin-indifferent). (3) The DNA of strain 3-str-td$_{(300)}$, which had originated from a transformation experiment, transferred both stable and unstable streptomycin-dependence. (4) All stable and unstable dependent colonies but one preserved the original level of resistance and dependence of the donor strain, whether or not the colonies had been selected on 30 or at 300 μg/ml streptomycin. The phenomenon of unstable dependence proved to be a transferable marker. DNA was prepared from an unstable dependent strain obtained in the transformation of streptomycin-dependence, and with this DNA the 3-str-s strain was transformed (Table II, exp. II/5). The transformation went on as a "normal" transformation of streptomycin-resistance, with a fequency of transformation characteristic of the transfer of the marker of streptomycin-resistance. Streptomycin-dependent transformants were not found among the colonies analyzed at random. All colonies were streptomycin-independent and showed the original level of streptomycin-resistance. Nevertheless, after underlaying streptomycin as described above, several new colonies were found. Their appearence proved that a fraction of the bacteria remained streptomycin-dependent (Table IV).

In the experiments carried out with a homologous DNA originating from a spontaneous dependent mutant of strain 3-str-s, (Table II, exp. II/53) the 24 hr transformation reaction resulted in a very low frequency of transformation. The transformation frequency of the 48 hr reaction was greater,

Table III

Character distribution of the transformants obtained in Exp. II/26 with the DNA of strain 3-str-td$_{(300)}$

Duration of the transformation reaction hrs.	CHARACTER OF THE TRANSFORMED COLONIES	Concentration of streptomycin(μg/ml) at which the transformants were selected						Sum
		30			300			
		Size of the transformed colonies						
		Small	Normal	Great	Small	Normal	Great	
24	Stable colonies (dependent on the original level	2	1	0	0	0	0	23
	Unstable colonies (dependent on the original level	3	9	0	1	7	0	
	Colonies resistant on the original level	1	30	3	0	19	2	57
	Colonies with lower level of resistance	2	0	0	0	0	0	
	Persistents	8	1	0	1	1	0	11
	Sum	16	41	3	2	27	2	91
48	Stable colonies (dependent on the original level	1	8	0	1	0	0	15
	Stable colonies with lower of dependence	0	1	0	0	0	0	
	Unstable colonies (dependent on the original level)	0	4	0	0	0	0	
	Colonies resistant on the original level	0	17	1	0	4	1	24
	Colonies with lower level of resistance	1	0	0	0	0	0	
	Persistents	0	2	0	0	0	0	2
	Sum	2	32	1	1	4	1	41

Recipient of DNA: strain 3-str-s.

Characterization of transformants was carried out on plates containing 0.30 or 300 μg/ml streptomycin.

0.024 per cent, between the frequencies obtained with the DNA of strain 3-str-td$_{(300)}$ (0.004 per cent) and that of strain 3-str-td$_{(300)}$ (0.064 per cent).

The increase in transformation frequency after 48 hrs seems to be contradictory to the result of experiment II/26 (Table II). Thus the question remains to be answered whether the greater number of transformants is due to their replication or is simply the result of the fact, that the 24 hr and 48 hr reactions were done in two separate parallels. As transformation was carried out in

Table IV

Streptomycin-dependent cells after transformation with the DNA of strain 3-str-td$_{\mu(300)}$

Dilution of culture	Original colony count in. 0.1 ml	Number of additional colonies, 0.1 ml	Additional growth, per cent
	29	10	34
	24	6	25
1 : 10,000	31	1	3.2
	27	1	3.2

After the appearence of streptomycin-independent colonies the agar layer was cut out, placed on a layer containing streptomycin (in an amount sufficient to a final concentration of 100 μg/ml of streptomycin in each layer), the position of the colonies was noted, then the plates were stored overnight at 4°C for diffusion of streptomycin, the plates were reincubated for 48 hrs.

a medium lacking streptomycin, only the streptomycin-indifferent transformants had been able to divide. Transformant type analysis demonstrated that the small and normal sized colonies appearing after 24 hr transformation reaction on media containing 30 or 100 μg/ml of streptomycin acquired the same level of streptomycin-dependence as the donor strain, while the colonies grown on 300 μg/ml of streptomycin proved to be resistant. The distribution of colonies on plates containing streptomycin in the 48 hr transformation reaction, is shown in Table V.

Apart from two resistant colonies, all transformants were stable dependent, with the same or a lower level of dependence than that of the donor. In this case the multiplication of resistant transformants could be excluded, as such transformants were not present. Thus, the conclusion had to be drawn that the seemingly contradictory results were due to the transformation occurring in separate parallels.

The lack of streptomycin does not permit replication of the streptomycin-dependent transformants, but does not hinder them to survive and later to become phenotypically manifest on media containing streptomycin.

In connection with the expression of the new phenotype, only preliminary experiments have been done. The time of addition of DNA, the duration of the transformation reaction, the time of addition of streptomycin to the transformation system *(i.e.* into the liquid medium) or the time of selection of the transformants on the agar medium containing streptomycin, was varied and the number and type of transformants was studied. The period of 8 or 16 hours was not sufficient for the expression of the marker, the first dependent colonies appeared after 24 hrs. (The experiments were carried out with unsynchronized cultures.)

The transformation was sensitive to deoxyribonuclease. In the controls without DNA, or with homologous DNA of strain 3-str-d$_{(1000)}$ and DN-ase,

Table V

Distribution of the transformants obtained in Exp. II/53 with the DNA of strain 3-str-d$_{(1000)}$

CHARACTER OF THE TRANSFORMED COLONIES	Concentration of streptomycin of the plates (μg/ml) at which the transformants were selected from								
	30			100			1000		
	Size of the transformed colonies								
	Small	Normal	Great	Small	Normal	Great	Small	Normal	Great
Stable colonies (dependent on the original level)	56	0	0	3	79	0	4	6	0
Stable colonies with lower of dependence	63	0	0	1	34	0	1	14	0
Total	119	0	0	4	113	0	5	20	0
Original level of resistance	0	0	0	0	0	0	0	0	0
Lower level of resistance	0	0	0	0	0	0	0	0	0
Total	0	0	0	0	0	0	0	0	0
Persistents	2	0	0	0	0	0	0	0	0
Total	121	0	0	4	113	0	5	20	0

Recipient: strain 3-str-s.
Further analysis of transformants was carried out on plates containing 30 or 300 μg/m streptomycin.

the frequency of colonies appearing on the selective medium was 0.000002 per cent for the variant without DNA, and 0.000008 per cent for the variant with DNA and DN-ase. All the appearing colonies were streptomycin-resistant. Streptomycin-dependent colonies were found with a frequency of 0.0006 per cent in variants with DNA not disrupted with DN-ase. The type distribution of transformants was the same as in other transformation experiments.

Discussion

In the experiments of streptomycin-dependence transformation, only strain 3-str-s proved to be a suitable recipient, *i.e.* the very strain which in the experiments of streptomycin-resistance transformation showed the highest frequency of transformation (0.5—1 per cent) [3]. The transformation of streptomycin-dependence has been reproduced in a number of experiments, but the frequency of transformation was always very low. Genetical causes may perhaps be responsible for this fact, namely that we are dealing with a very extended complex locus. In this case the mutation responsible for streptomycin-dependence would be in the same locus as the complex streptomycin-

resistance mutation. Synapsis, integration and recombination of the entire locus which carries the regions of resistance and dependence, might be hindered because of the shortness of the homologous regions. The probability of the role of homology is suggested by the different frequencies of transformation obtained with different DNA donors. The DNA donors used were the transformant strains obtained after the transformation of streptomycin-dependence. The transformation frequencies were higher in every case than those resulting from transformations with non-homologous DNA (Table II).

The arrangement of streptomycin-dependence and resistance in one complex locus is supported by the following additional facts.

(1) In the transformation experiments carried out with one and the same DNA, only part of the appearing transformant colonies was streptomycin-resistant. The other part was streptomycin-dependent (transformations with DNA originating from strains 1-str-$d_{(1000)}$ (Fig. 1); 3-str-$d_{(1000)}$ (Fig. 2); 3-str-$td_{(300)}$ (Fig.1); 3-str-$td_{\mu(300)}$ (Fig. 1).

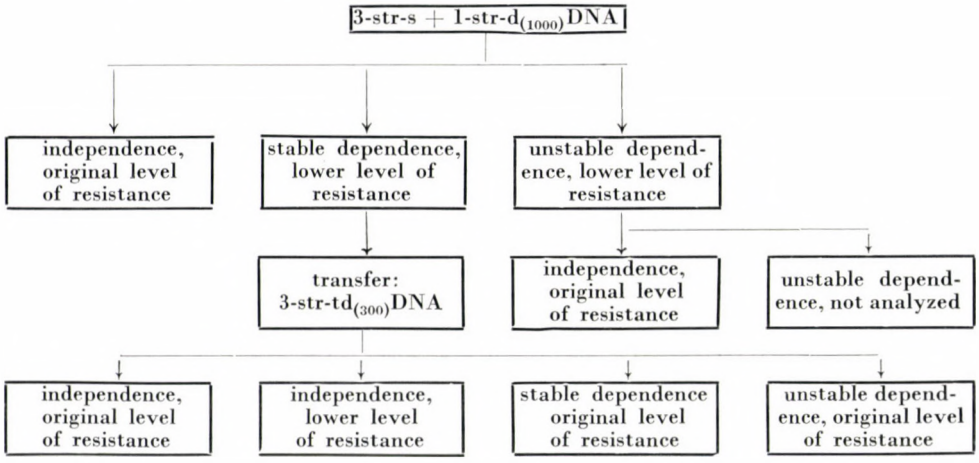

Fig 1. Identified types of transformants obtained in transformation of streptomycin dependence with the DNA from the streptomycin-dependent strain l-str-$d_{(1000)}$

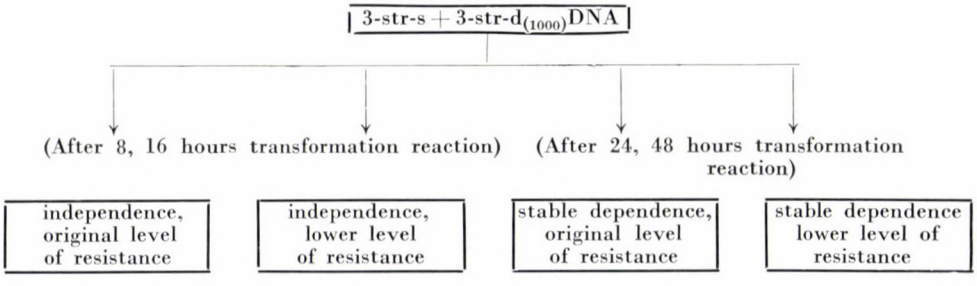

Fig. 2. Transformation by DNA obtained from the streptomycin-dependent strain 3-str-$d_{(1000)}$

(2) Transformation with DNA of strain 1-str-$d_{(1000)}$ resulted in stable and unstable streptomycin-dependent transformants. The unstable dependence proved to be a transformable marker (Fig. 1).

(3) The DNA of the stable streptomycin-dependent 3-str-$td_{(300)}$ strain, obtained during a transformation to streptomycin-dependence transferred stable and unstable streptomycin-dependence (Fig. 1).

The phenomenon of unstable dependence may be explained in several ways. (a) The rate of back mutation of streptomycin-dependence to streptomycin resistance may be very high. (b) The rate of division of the cells which have integrated the streptomycin-dependent region of the donor-DNA is lower than of those cells, which had received the streptomycin-resistant region, therefore the streptomycin-dependent cells were overgrown by the streptomycin-resistant ones in a heterogeneous population. (c) Unstable dependent transformants are in the possession of the integrated resistance mutation, accompanied by the dependence mutation in a form which is easily lost. This might explain the possibility of continuous segregation of the transformants [1]. (d) Mutation at another (suppressor) locus may be responsible for "reversion" to independence. (e) The phenomenon described reminds one of abortive transduction (RYAN, personal communication).

(4) In the transformation of streptomycin-dependence the majority of streptomycin-resistant colonies was found on plates containing a low concentration of streptomycin. However, in the course of further analysis part of the transformants proved to have the same level of streptomycin requirement, as the donor (original level of dependence), whereas the rest required less streptomycin ("decreased" dependence). For example the transformant strains 3-str-$td_{\mu(300)}$ and 3-str-$td_{(300)}$ originated from transformation experiments, where the DNA donor was the strain 1-str-$d_{(1000)}$.

(5) In the transformation of streptomycin-dependence, in those cases where the streptomycin-resistance had been transferred, part of the transformants obtained the original level, while the other only a decreased resistance.

The type of streptomycin-dependence is in some way connected with the size of the colonies, in other words with the rate of growth. Large colonies were always streptomycin-independent; streptomycin-dependent colonies were found only among the small colonies or those of normal size. This phenomenon may be due to some defect in the biochemical pathway.

The identified types of transformants obtained in the course of streptomycin-dependence transformation may be summarized after BALASSA [1] with modifications in Fig. 1 and 2.

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THIN LAYER AND GAS CHROMATOGRAPHIC ANALYSIS OF SYPHILIS ANTIGENS

By

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Summary. The phosphatide composition and fatty acid content of syphilis antigens with varying serological activity have been examined.

(i) Thin layer chromatography revealed the presence of 6 different components in the syphilis antigens.

(ii) Suitable and unsuitable antigens differed mainly in containing various amounts of a substance designated as component 6. This factor was found in smaller amounts in unsuitable antigens.

(iii) The serological reactivity of unsuitable antigens improved when component 6 was added; however, higher amounts of this substance caused an aspecific reaction.

(iv) The fatty acid content of the antigens amounted to about 50 per cent. Oleic, linolic and stearic acids were considered to be partly responsible for serological activity.

(v) Antigens with varying serological activity differed considerably in their saturated and unsaturated C_{18} fatty aid content and in the distribution of these substances. The oleic: linolic acid proportion was over 1.500 in suitable and under 1.000 in unsuitable antigens.

(vi) The usefulness of thin layer and gas chromatography in the control of antigens is discussed.

In recent years much progress has been made in lipid research. Thin layer [1, 2] and gas chromatography [3, 4] allowed intensive investigations into the structure of complex lipids including syphilis antigens. Knowledge of the composition of the latter products is desirable for the preparation of highly pure and specific antigens, and for the elucidation of decomposition processes occurring during preparation and storage. The practical side of the problem is that the serological activity of such preparations decreases on prolonged storage in alcoholic solution (5).

Materials and methods

Antigens with varying serological activity were prepared by alcoholic extraction from acetone and ether treated beef heart. Suitable and unsuitable Kahn and Citochol antigens were also tested. In order to avoid errors due to a degradation process occurring in antigen systems, preparations approximately of the same age were employed. The antigens were subjected to thin layer and gas chromatography and serological examination.

Apparatuses. (A) The apparatus devised by the authors allowed a reproducible preparation of layers of required thickness*. (B) Pye Argon chromatograph supplied with electronic integrator.

Reagents. (a) Thin layer chromatography: Silicagel G (Merck, Darmstadt), phosphomolybdic acid (Merck, Darmstadt), ninhydrin, chloroform, methanol, ether (Reanal, Budapest);

* To be published; applied for patent.

(b) Column chromatography: Silicagel, 0.2—0.5 mm (Merck, Darmstadt), chloroform, methanol, ethanol distilled from ferrous sulphate (Reanal, Budapest); (c) Gas chromatography: Celit 545 (100—120 mesh) polyethylene-glycoladipate (PEGA), Apieson L (APL) (Applied Science Lab., U. S. A.), Argon gas, 99.95 per cent purity (Nitrogen Works, Pét).

Preparation of thin layers was carried out with Silicagel G on 6×20 cm plates. Activation lasted for 10 to 15 minutes at room temperature, then for 1 hour at 110°C.

In experiments performed with layers 100, 150, 200, 250, 300, 500, 750 and 1000 μ thick, the best separating capacity was obtained with the 500 μ layer (Fig. 1).

Preparation of antigens. Antigens stored in 95 per cent ethanol were evaporated to dryness in nitrogen at reduced pressure. The preparations were dried to constant weight over phosphorus pentoxide in the vacuum exsiccator. Then 2 per cent stock solutions were prepared from the dried antigens with 95 per cent ethanol. Finally from each antigen 400 μg amounts were placed onto the plates.

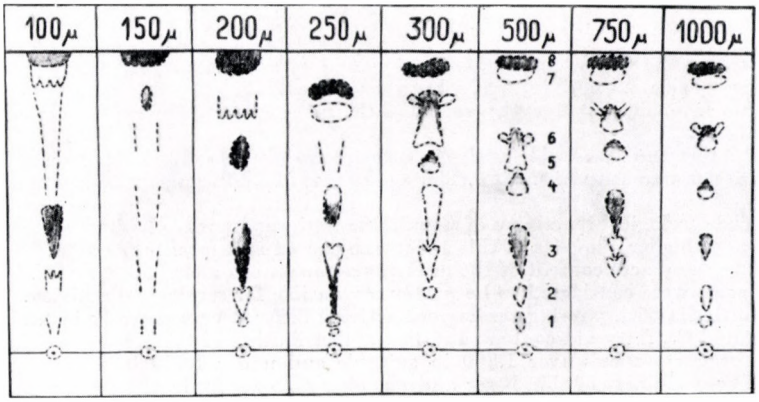

Fig. 1. Separating capacity of Silicagel layers of different thickness. Chloroform: methanol: water (62 : 25 : 4) solvent. Development at room temperature. Detection of spots with 5 per cent alcoholic phosphomolybdic acid

Solvent system. Of the examined solvents [6, 7] the chloroform: methanol: water (65 : : 25 : 4) mixture described by WAGNER and WAGNER *et al.* [2, 8] gave the most satisfactory results. The chromatograms were developed by the ascending technique at room temperature for 50 to 60 minutes.

Detection of spots was carried out with the following reagents. (a) MANGOLD's [9] 5 per cent alcoholic phosphomolybdic acid, a general lipid reagent; (b) ninhydrin reagent modified by MOFFAT and LYTLE [10] for detecting amino group-containing phosphatides; DRAGENDORFF's reagent [9] for showing lecithin-type components.

Column chromatography was performed through 40×2 cm columns containing 23.5 g. silicic acid (0.2—0.5 mm). As eluent, chloroform : methanol : water (65 : 25 : 4) was used. After evaporating to dryness, 200 mg aliquots of the antigens were redissolved in small volumes of ethanol and poured on the columns. With the above eluent, 36 fractions, each amounting to 2.5 ml, were separated. The first 4 fractions were discarded; susbsequent fractions were separately evaporated and subjected to thin layer chromatography. Thus various components were distinguished in the antigens. Component 6 eluted in pure solution in fractions 10, 11 and 12. Fractions containing this substance were united, evaporated to dryness and added in 0.1 per cent solution to unsuitable antigens.

Gas chromatography. Column (a): Apieson L, 100—120 mesh Celit 545, at 200°C; Column (b): 10 per cent polyethylene-glycoladipate, 100—120 mesh Celit 545, at 178°C, in glass columns 122 cm long; flow velocity of carrier gas, 40—60 ml/min; detector current, 1250 V; travelling speed of recording paper, 15.24 cm/hour. Quantitative analysis was performed by means of an automatic electronic integrator without employing calibration factors. In order to identify fatty acids with unsaturated bonds, one of the preparations was hydrogenized by POUKKA's method [11].

Preparation of antigens for gas chromatography. Antigens dissolved in 95 per cent ethanol were evaporated in inert atmosphere and dried to constant weight over phosphorus pentoxide.

Extraction of fatty acids. Dried antigens were hydrolysed under a reflux for 4 hours in 15 ml 20 per cent potassium hydroxide dissolved in a mixture containing equal volumes of water and methanol. After cooling fatty acids were liberated with analytical grade hydrochloric acid diluted 1:3, then shaken out 3 times with ethyl ether. The united ether extracts were evaporated in inert atmosphere then dried over phosphorus pentoxide to constant weight.

Conversion of fatty acids to methyl esters was carried out with diazomethane prepared freshly from nitrosomethylurea [12]. The obtained fatty acid methyl esters were gas chromatographed.

Results

Results of thin layer chromatography. As mentioned above, antigens approximately of the same age were examined.

(a) Figs 2,3 and 4 show thin layer chromatograms of 3 different antigens prepared by alcoholic extraction. The chromatograms were developed by the ascending technique in a 500 μ Silicagel G layer with chloroform: methanol: water (65 : 25 : 4). Spots were detected with phosphomolybdic acid.

Fig. 2 shows the thin layer chromatogram of serologically unsuitable antigens 16 a—16 e. All antigens contained 6 components. Spots 1 represented

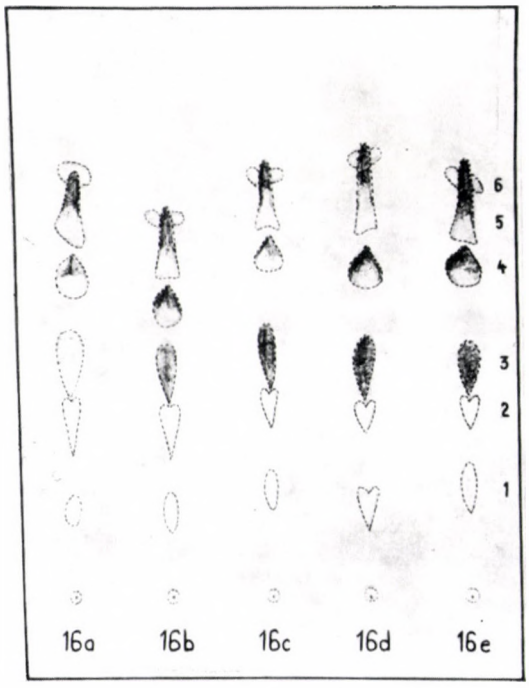

Fig. 2. Thin layer chromatogram of syphilis antigens 16a — 16e. Components: 1 = lysolecithin, 2 = sphingomyelin, 3 = lecithin, 4 = cholamine cephalin, 5 = cardiolipin, 6 = unknown. Serologically unsuitable antigens

lysolecithin components giving positive colour reactions with DRAGENDORFF's reagent and phosphomolybdic acid ($R_f = 0.17 - 0.23$). Spots 2 giving colour reaction with phosphomolybdic acid, DRAGENDORFF's reagent and ninhydrin, corresponded to sphingomyelin components ($R_f = 0.30 - 0.34$). Spots 3 represented lecithin components and gave colour reaction with DRAGENDORFF's

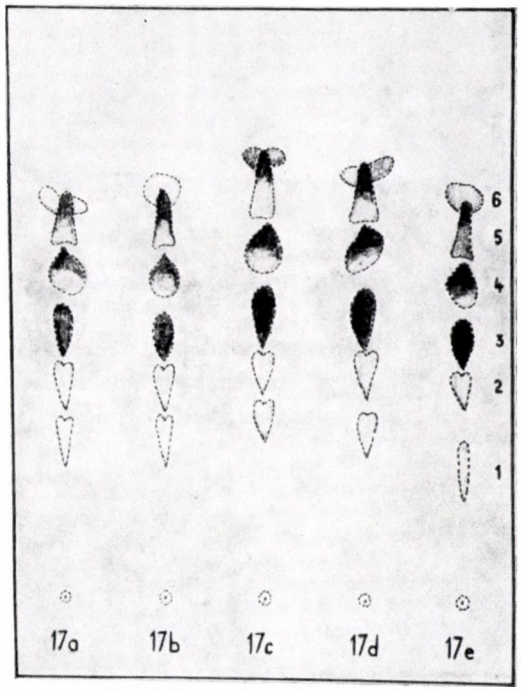

Fig. 3. Thin layer chromatogram of syphilis antigens 17a — 17e. Components: 1 = lysolecithin, 2 = sphingomyelin, 3 = lecithin, 4 = cholamine cephalin, 5 cardiolipin, 6 = unknown. Serologically suitable antigens

reagent and phosphomolybdic acid ($R_f = 0.40 - 0.44$). Spots 4 reacting with phosphomolybdic acid and ninhydrin corresponded to cholamine cephalin components ($R_f = 0.57 - 0.63$). Spots 5 were cardiolipin components reacting with phosphomolybdic acid only ($R_f = 0.80 - 0.88$). Spots 6 have not been identified. They reacted only with phosphomolybdic acid and their R_f value was approximately 0.90.

Fig. 3 shows the thin layer chromatogram of the serologically suitable antigens 17 a—17 e. In the number and identity of their components these antigens resembled the unsuitable preparations, but contained a higher amount of the unidentified component 6.

Fig. 4 shows the chromatogram of the serologically unsuitable antigens 19a and 19b. Spots 1—5 of antigen 19 were identical with those of antigens 16 and 17. Component 6 was absent from antigen 19.

(b) Thin layer chromatograms of suitable and unsuitable Kahn and Citochol antigens (not presented here) gave the following results. All antigens contained lysolecithin, sphingomyelin, lecithin, cholamine chephalin and cardiolipin components. The antigens differed only in their component 6 content. This component was present in suitable Kahn and

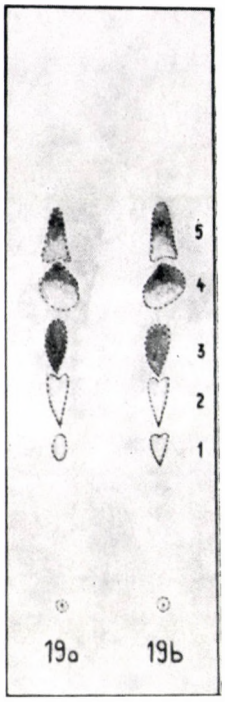

Fig. 4. Thin layer chromatogram of syphilis antigens 19a and 19b. Components: 1 = lysolecithin, 2 = sphingomyelin, 3 = lecithin, 4 = cholamine cephalin, 5 = cardiolipin, Serologically unsuitable antigens

Citochol antigens in amounts corresponding to the serologically suitable preparation 17. The unsuitable Kahn antigen contained 6 amounts corresponding to the serologically unsuitable antigen 16.

(c) Thin layer chromatography was carried out also with pure cardiolipin, heart lecithin and egg lecithin. Spot 6 was not recorded in either of these chromatograms. Component 6 should be considered as a decomposition product, the presence of which in complex lipids may indicate decomposition, oxidation, degradation, etc. processes.

In subsequent experiments it has been examined whether component 6, when added to unsuitable antigens, altered the chromatogram and improved the serological activity. Component 6, which had been separated on Silicagel

Table I

Serological reactions of antigens supplemented with component 6

No.	Antigen	Component 6 added, μg	Result of flocculation reaction	
			positive serum	negative serum
1	Unsuitable	—	+	±
2	Unsuitable	50	++	+
3	Unsuitable	100	++	++
4	Unsuitable	200	+	+++
5	Suitable	—	++++	—

column from a suitable antigen, was added to unsuitable preparations in 50 to 200 μg amounts. The antigens were then examined for flocculation reaction with positive and negative sera. A suitable and an unsupplemented unsuitable antigen served as control. It has been revealed that both the specific and the aspecific sensitivity of the supplemented antigen increased. Addition of higher amounts of component 6 resulted in complete aspecificity.

Table I clearly indicates that component 6 is not the only factor responsible for the weak reactivity of unsuitable antigens. In view of this finding, the fatty acid spectrum of the antigens was examined by chromatography.

Result of gas chromatography. Table II presents the total fatty acid content of antigens with different serological activity. The total fatty acid content of suitable and unsuitable antigens was about 50 per cent.

Table II

Total fatty acid and content of syphilis antigens with different serological activity

Antigen	Saponified material, g	Fatty acid	
		g	per cent
16 a	0.1652	0.0843	51.02
16 b	0.1452	0.0772	53.16
16 c	0.1343	0.0697	51.89
16 d	0.1605	0.0820	51.09
16 e	0.1010	0.0510	50.49
17 a	0.1888	0.1025	54.29
17 b	0.1627	0.0845	51.93
17 c	0.1689	0.0858	50.72
17 d	0.1529	0.0756	49.44
17 e	0.1348	0.0620	45.99
19 a	0.1546	0.0855	55.30
19 b	0.1341	0.0709	52.80

Table III

Fatty acid composition of syphilis antigens+

No.	Fatty acids	Abbreviated designation	Per cent of area		
			Antigen 16*	Antigen 17*	Antigen 19*
1	Oleic acid	$C_{18:1}$	22.83	*33.08*	23.12
2	Linolic acid	$C_{18:2}$	*39.31*	18.31	*34.81*
3	Palmitic acid	$C_{16:0}$	31.76	21.64	35.17
4	Stearic acid	$C_{18:0}$	—	25.07	1.33
5	Methyl tetradecanoic acid	i—C_{15} or ai—C_{15}	3.9	2.39	5.59
6	Palmitoleic acid	$C_{16:1}$	+	+	+
7	Methyl pentadecanoic acid	i—C_{16} or ai—C_{16}	+	+	+

+ Results obtained on PEGA columns; no calibration factors were used. Area percentage values corresponded to M% in ± 0.5%.

* Average value of antigens 16a, b, c, d, e; limits of error, ±3%.

** Average value of antigens 17a, b, c, d e; limits of error, ±3%.

*** Average values of antigens 19a, b; limits of error, ±3%.

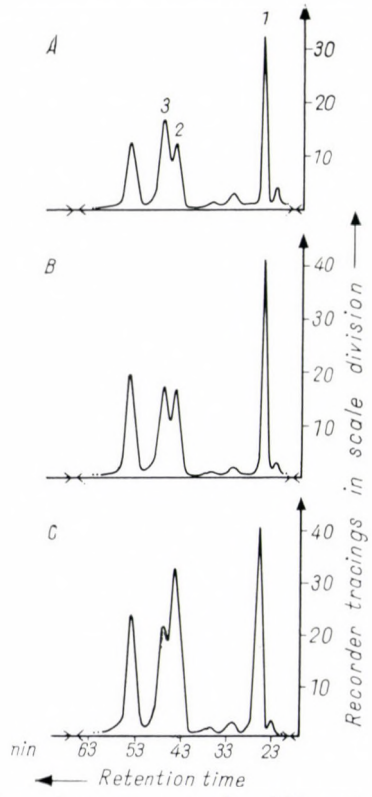

Fig. 5. Characteristic parts of gas chromatograms of syphilis antigens with different serological activity. A = suitable Kahn antigen, B = Meinicke antigen, C = unsuitable Kahn antigen. Fatty acids: 1 = palmitic acid, 2 = linolic acid, 3 = oleic acid

Table III shows that the examined antigens contained 6 or 7 different fatty acids. The main component in the serologically unsuitable antigen 16 was linolic acid; palmatic acid was next in order. The predominating fatty acids in the serologically suitable antigen 17 were oleic and stearic acid. In the unsuitable antigen 19 palmatic and linolic acid predominated. Antigens with varying serological activity differed considerably in the occurrence of fatty acids containing 18 carbon atoms.

Table IV

Oleic acid: linolic acid ratio in syphilis antigens

Antigens	Oleic acid: linolic acid	Mean
16 a	0.526	
16 b	0.593	
16 c	0.482	0.5938
16 d	0.501	
16 e	0.867	
17 a	1.658	
17 b	1.742	
17 c	1.520	1.8404
17 d	1.989	
17 e	2.293	
19 a	0.704	0.6645
19 b	0.625	

Fig. 5 shows characteristic parts of gas chromatograms prepared with three biologically different antigens. It is seen that the fatty acid spectrum of the examined antigens differed mainly in C_{18} fatty acids.

The oleic acid: linolic acid ratio for the suitable antigens 17a—17e was over 1.500. For unsuitable antigens values of 0.600 or so were obtained (Table IV). This finding indicates that a proper oleic acid: linolic acid ratio might be essential for the specificity of the serological reaction, independently of the fact that the antigens differed significantly in stearic acid content. No explanation can be offered at present for the latter finding. According to FAURE [13] saturated fatty acids play a secondary part in the serological activity of antigens.

Discussion

The purpose of the present work was to show whether thin layer and gas chromatography were suitable for revealing differences among various syphilis antigen preparations. As the antigens prepared under identical conditions

differed considerably in serological activity, it was desirable to identify the components of our products. The unsuitability of some preparations might be due to technical errors or qualitative differences in the beef heart used as raw material. Our experiments have shown that syphilis antigens with different serological activity differ in the content of an unidentified factor designated as component 6. In suitable antigens this substance was present in higher amounts than in unsuitable preparations.

When a purified preparation of component 6 was added to serologically unsuitable antigens, it was not decomposed or bound to other components, and therefore it appeared separately on the chromatograms. It may be assumed that this factor is a cardiolipin decomposition product. The ratio between this factor and the other components of the antigen influences the specificity of the serological reaction. For the identification of component 6 further investigations are needed.

The degradation of cardiolipin has been studied by FAURE and MORELEC [5] and FAURE [13] who could show that on prolonged storage in alcoholic solution, cardiolipin undergoes a structural alteration resulting in a decrease of serological activity. Two kinds of changes have been observed: degradation of the phosphatide molecula and oxidation of fatty acids. Serological activity is impaired by both processes, especially by the latter: the higher the degree of oxidation, the weaker the serological activity.

Our studies also indicated that suitable and unsuitable antigens differ primarily in the degree of saturation of C_{18} fatty acids. It may be assumed that a certain oleic acid content is required for suitable serological activity. When the oleic acid: linolic acid ratio was calculated for a suitable Kahn antigen, the obtained value exceeded 1.500. With a less active Citochol antigen this value was about 1.000, while with an unsuitable Kahn antigen, similarly to the ratio obtained with the unsuitable antigens presented in Table IV, it was about 0.600. This observation indicates that C_{18} fatty acids containing 1 or 2 unsaturated bonds, influence the result of the serological reaction.

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ALKALINE PHOSPHATASE REPRESSION BY INORGANIC PHOSPHATE IN BACILLUS ANTHRACIS AND BACILLUS CEREUS

By

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Summary. Alkaline phosphatase production by *Bacillus anthracis* and *B. cereus* in a semisynthetic medium containing defined amounts of phosphate, has been examined. At low concentrations of inorganic phosphate both species formed alkaline phosphatase. Due to a repression by inorganic phosphate, at higher phosphate concentrations the organism failed to produce the enzyme.

Detection of phosphatase for the characterization of certain pathogenic bacteria was first recommended by BRAY and KING in 1943 [1]. The reaction is particularly important in the examination of *Micrococcus pyogenes* var. *aureus*, as the presence of phosphatase in this organism may be associated with virulence and penicillin resistance [2—4]. Phosphatase activity of *M. pyogenes* is generally determined on complex media containing peptone, broth, yeast extract, etc. Some authors recommended substrates (phenolphthalein phosphate or p-nitrophenyl phosphate) for the test which yields coloured decomposition products in colonies developed on nutrient agar plates [2, 6]. Others used washed-off bacterial suspensions for quantitative phosphatase tests [4, 5]. The phosphatase test seems also to allow differentiation between *B. anthracis* and *B. cereus*. When peptone-yeast extract agar is used, phenolphtalein phosphate is split by *B. cereus* but not by *B. anthracis* [6].

Phosphatase activity in *E. coli* was first investigated by HORIUCHI *et al.* [7] and by TORRIANI [8]. It has been shown that the phosphate content of the medium highly influences the enzyme production, which is gradually repressed at increasing phosphate concentrations. Some mutants have been shown, which are able to produce phosphatase freely at higher phosphate concentrations (constitutive mutants) [9]. According to KNO and BLUMENTHAL, non-repressible strains were common among laboratory collection cultures of *E. coli* and *M. pyogenes* [10].

Findings that phosphatase production in some bacterial species may be repressed by inorganic phosphate, indicate that in carrying out the test, cultural conditions should strictly be defined. It should be kept in mind that the phosphate content of complete media prepared from natural ingredients may be highly variable. Phosphate may be present at inhibitory concentrations in such media. Therefore, we thought advisable to reinvestigate our results

obtained with *B. anthracis* and *B. cereus* [6]. For this purpose the bacteria were cultivated on semisynthetic media containing well-defined concentrations of phosphate. Both organisms exhibited suboptimal growth in the presence of protein hydrolyzate, salts and glucose [11, 12]. Growth could be enhanced with some accessory substances, especially with thiamine. The casein hydrolyzate medium [13] applied previously by one of the present authors seemed unsuitable in view of the considerable phosphate content of casein hydrolyzate. Therefore, a commercial lactalbumin preparation was employed as an amino acid source.

Materials and methods

Strains. Properties of *B. anthracis* and *B. cereus* strains maintained in the collection of the Institute of Microbiology were described in a previous paper [6].

Medium. The double-strength medium contained the following ingredients: L-glutamsc acid, 2 g; lactalbumin hydrolyzate (Difco), 2 g; NaCl, 6 g; KCl, 2 g; Na_2SO_4 (dried), 1 g; Trii (hydroxymethyl) amino methane, 4 g; distilled water, 1000 ml; pH 7.2. To the medium the following solutions were added: 1 per cent ferric ammonium citrate, 1 ml; 1 per cent $MnSO_4 \cdot 4H_2O$, 0.5 ml; 1 per cent $MgSO_4 \cdot 7H_2O$, 20 ml. Sterilization was carried out at 110°C for 30 minutes.

Before use the medium was diluted with an equal volume of distilled water or with a solution containing 3 per cent washed agar. Then to 100 ml portions 2 ml 20 per cent glucose was added. Phosphate ion concentration in the medium varied between 30 and 50 μM.

The medium ensured suboptimal growth, which could be somewhat enhanced by increasing the phosphate concentration. However, even at a sufficient phosphate concentration the growth was not optimal. The growth of *B. anthracis* in synthetic medium can be "stimulated" by the addition of nuclein bases or vitamins [11—14]. Therefore the solid medium was supplemented with nucleic bases and 10 different vitamins. The substances and their effective concentration have been described previously [13]. The supplemented medium will be referred to as lactalbumin medium.

Cultivation. Different amounts of K_2HPO_4 to give 50 to 500 μM phosphate concentrations were added to 10 ml portions of the medium. In addition, the medium contained 30 to 50 μM phosphate originating from the phosphate contamination of the ingredients.

Precultivation was performed overnight at room temperature in 10 ml medium containing 500 μM phosphate. The medium was inoculated with one drop of yeast extract-peptone medium culture. The pre-culture was washed twice in saline and resuspended in the original lactalbumin medium. Then 20 ml aliquots of lactalbumin to which different amounts of phosphate had been added, were dispensed in 100 ml side tubed Erlenmeyer flasks. Each flask was inoculated with 0.2 ml suspension. The flasks were shaken in a 37°C water bath for 5—6 hours. Quantitative phosphatase determinations were performed from these cultures.

Quantitative determination of phosphatase. The cultures were centrifuged, washed twice in saline and resuspended in 0.2 *M* pH 8.7 tris-HCl buffer. Protein content was determined by use of the Folin reagent as desribed by MILLER [15].Crystalline serum albumin was used as a reference. The enzyme activity resulting in the decomposition of 1 μM/ml p-nitrophenyl phosphate at 37°C in 30 minutes was regarded as the unit. The specific activity for 1 mg protein was calculated from the obtained values and from the protein content of the suspension.

Detection of phosphatase production on solid medium. The calculated amount of phosphate was pipetted at 1 ml volumes into Petri dishes, then plates were poured with 19 ml portions of lactalbumin agar medium. Thus a series of plates with increasing phosphate concentration was prepared. Each plate was divided into 8 sectors and seeded with 8 different cultures by the needle point or streak technique. The plates were incubated for 48 hours at 37°C, then 0.1—0.5 per cent p-nitrophenyl phosphate in tris-buffer of pH 8.5 was dropped on the colonies. Readings were made after 10 minutes. When the substrate was applied within the concentration range, phosphatase activity was indicated by an intensive yellow colour of the colonies. On the basis of the colour intensity, different grades of positivity could be scored. Cultures not turning yellow 30 minutes were regarded as negative.

Results

Different variants of *B. anthracis* and one *B. cereus* strain grown at various phosphate concentrations were examined for specific phosphatase activity. The results are shown in Fig. 1. When no K_2HPO_4 was added separately to the medium,only phosphates originating from the lactalbumin hydrolyzate were present (30—50 μM). In the experiments acapsulogenic *B. anthracis* strain Vollum (VC^-) and its two variants differing in colonial morphology

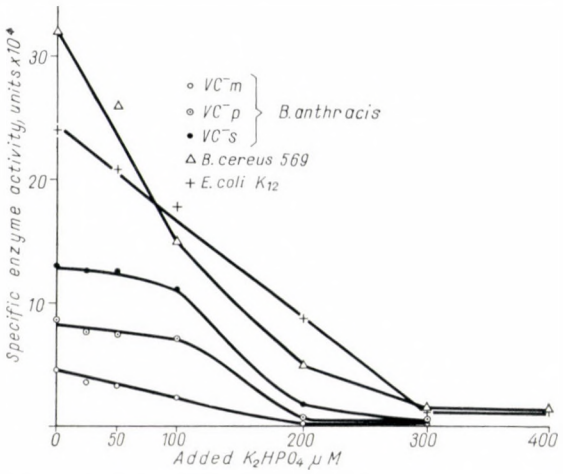

Fig. 1. Alkaline phosphatase activity of *B. anthracis*, *B. cereus* and *E. coli* cells grown at different phosphate concentrations

were used. The young colonies of one of these variants had a mosaic-like structure; the other variant produced uniformly compact colonies. The parent culture contained both colonial variants. As seen in Fig. 1, the two isolates differed considerably as to phosphatase production. The parent culture (VC^-p) occupied an intermediary position. It is interesting that repression increased rapidly and considerably over 100 μM phosphate concentration. *B. cereus* strain 569 and *E. coli* strain K12 produced higher amounts of phosphatase than the examined *B. anthracis* cultures. Repression in the former strains was evident at low phosphate concentrations; these cultures, however, were able to exert a minimum phosphatase activity even in the presence of large amounts of phosphate (over 300 μM).

Thus repression of phosphatase in the examined strains grown in liquid cultures at various phosphate concentrations, was evident. Synthesis of the enzyme was inhibited by inorganic phosphates incorporated in the medium. Studies on a larger number of cultures were performed by a semi-quantitative

plate technique. Lactalbumin agar plates containing different concentrations of phosphate were seeded with the strains to be examined. After incubation for 72 hours at 37°C, 0.5 per cent p-nitrophenyl phosphate was dropped over large colonies. The degree of phosphatase activity was estimated from the intensity of the colonies' yellow colour. Table I shows the reaction of 12 *B. anthracis* strains grown at various phosphate concentrations. The colonies of two strains (72 and 100) took on an uncertain or very pale yellow (±) colour even at considerable phosphate concentrations. In other cultures *(e.g.* in NPC^-) a definite repression occurred at low concentrations. Except for strain 72, complete repression was revealed in all cultures at 500 μM phosphate concentration.

Table I

Phosphatase reaction of macrocolonies of B. anthracis strains grown on lactalbumin hydrolyzate agar containing various concentrations of inorganic phosphate

Strain	Additional phosphate μM					
	0	50	100	200	300	400
Davis	N. T.	N. T.	++	+	±	—
VC^-	++	+	—	—	—	—
VC^+	++	+	—	—	—	—
NPC^-	++	+	±	—	—	—
35—18	++	—	—	—	—	—
66	++	+	+	+	±	—
69 S	++	+	+	—	—	—
72	++	+	±	±	±	±
74R	N. T.	N. T.	++	+	—	—
99R	N. T.	N. T.	++	++	+	—
100	++	+	+	±	—	—
102	++	+	+	±	—	—

N. T. = not tested; ++ = intensive yellow colour; + = yellow colour of moderate intensity; ± = very pale yellow colour or uncertain reaction.

Repeated experiments yielded similar results. The same findings were obtained when the minimal lactalbumin hydrolyzate medium was supplemented with nuclein bases and vitamins. Cultivation time (72 or 96 hours) and substrate concentration (0.1 or 0.5 per cent) exerted no influence on the results.

Experiments with 9 different *B. cereus* strains are summarized in Table II.

It is seen that all strains gave a strong phosphatase reaction at an added phosphate concentration as high as 200 μM. Although not indicated in Table II ++ or + reactions of *B. cereus* were generally more intensive in colour than those of *B. anthracis*. At high phosphate content, however, repression of

Table II

Phosphatase reaction of macrocolonies of B. cereus strains grown on lactalbumin hydrolyzate agar containing various concentrations of inorganic phosphate

Strain	Additional phosphate μM				
	100	200	300	400	500
No 2	++	++	+	—	—
No 4	++	++	+	+	—
No 5	++	++	+	±	—
No 5/P3	++	++	++	+	±
No 5/P13	++	++	++	+	±
No 6	++	++	—	—	—
No 116	++	++	++	+	+
WS	++	++	+	—	—
No 130	++	++	+	+	±

phosphatase production occurred also in *B. cereus*. A comparison of the data in Table I and Table II, reveals that repression of phosphatase production by inorganic phosphate was weaker in *B. cereus* than in *B. anthracis*.

The findings that *B. cereus* produces phosphatase more actively than *B. anthracis* and that the two organisms differ in the repression of enzyme production by phosphate, indicate that under the given experimental conditions the phosphatase test is a valuable means for differentiating between the two closely related species.

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A RAPID DIAGNOSTIC METHOD FOR THE IDENTIFICATION OF ENTERIC PATHOGENS

FLUORESCENT MICROAGGLUTINATION

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Summary. A rapid diagnostic method has been elaborated for the direct identification of enteric pathogens in faecal samples. The diluted faeces of patients suffering mainly from acute diseases were mixed with immune sera. The resulting microagglutination of bacteria was examined after acridine-orange staining under the fluorescent microscope. Flaky clumps of cells showing green fluorescence were easily distinguishable from orange coloured amorphous faecal constituents. As these formations were present in the control smear, too, their occasional green fluorescence caused no diagnostic difficulties.

Sensitivity and specificity of the method were checked by parallel culturing.

Five hundred faecal samples originating from patients hospitalized with enteritis and further 509 specimens from persons involved in a dysentery outbreak were examined. The former samples were positive in 23.2 per cent by cultivation and in 50.8 per cent by fluorescent microagglutination. In the material from the outbreak *Sh. flexneri* was cultured in 33.3 per cent, while the rapid method gave positive results in 59.1 per cent. The advantage of the new method is that diagnosis can be established within one hour and thus the patients can be admitted without delay to the ward designed for diseases caused by the corresponding pathogenic agent.

Ten thousands of cases caused by shigellae, salmonellae and pathogenic coli strains and the seasonal overcrowdedness of enteric departments indicate that sporadic and epidemic infections present considerable tasks for the routine bacteriologist. Epidemic and hygienic preventive measures somewhat decrease the rapid spread of pathogenic agents. However, the high frequency of carriers and in most enteric diseases the lack of effective vaccines mean a constant danger for the environment.

In order to prescribe an aimed individual treatment and to take effective measures against the spread of infection, early diagnosis is of utmost importance. By the use of classical bacteriological methods at least 24, but generally 48 to 72 hours elapse until a final diagnosis can be made. Cultural methods often fail to give positive results because the causative agent is destroyed during transport of the specimen or in consequence of previous drug treatment. In view of the above considerations, a rapid diagnostic method has been elaborated which allows direct identification of both living and killed cells.

Materials and methods

Faecal samples, depending on their consistence, were diluted 1 : 5 — 1 : 10, then sedimented by a low speed centrifugation (100 r. p. m.). When the sample was too fluid or contained mucus or blood, to a 2 ml portion 1 drop of 15 per cent NaCl was added in order to

assure a proper electrolyte concentration. One drop of the supernatant of the sedimented sample was deposited on a slide and mixed with one drop of Lugol solution diluted 1 : 3. Lugol solution served partly for alkalizing the sample, partly for effectively decreasing the autofluorescence of undigested faecal constituents. The reagent exerted no appreciable influence on the specific fluorescence of the agglutinated bacteria. To the supernatant — Lugol mixture one drop of diagnostic immune serum diluted 1 : 5 was added. After mixing thoroughly, the slide was left to stand for 20 minutes at room temperature. The suspension was then stained with pH 6 acridine orange (1 : 10,000) for 5 minutes at room temperature. Then a large cover glass was placed over the preparation and the excess fluid was blotted with filter paper. Readings were performed with a Leitz Model Ortholux II fluorescent microscope at ×250 magnification and blue light. Homologous reaction were indicated by green fluorescence of clumped bacteria; other constituents appeared in organe colour on light brown background. Examination of faecal samples from infants was carried out on 3 slides with polyvalent coli sera I, II and III. Faeces obtained from patients over one year of age were examined on 4 slides with polyvalent Salmonella C and H and with *Sh. flexneri* and *Sh. sonnei* diagnostic sera. When specific microagglutionation was observed, the positive result was immediately reported.

Results

The results yielded by fluorescent microagglutination and cultivation were compared by examining 500 faecal samples from patients admitted to the department of enteric diseases and 509 further faecal samples collected during a mass outbreak caused by *Sh. flexneri.*

The first group of non-selected materials obtained during the summer months was cultured by use of conventional bacteriological methods. On the day of arrival in the laboratory, each specimen was examined by the fluorescent microagglutination method. Between the rapid method and 48 hour cultivation there was a significant difference only as to the positivity for shigellae.

Table I indicates that the diagnosis was established in 23.2 per cent by cultivation and in 50.8 per cent by fluorescent microagglutination. The cause

Table I

Results obtained at cultivation and fluorescent microagglutination in the case of hospitalized patient with gastrointestinal symptoms

Method		Number of			Salmonella	Sh. flexneri	Sh. sonnei	Pathogenic coli
		examined	negative	positive				
		samples			positives			
Cultivation	No.	500	384	116	18	25	12	61
	%	100	76.8	23.2	3.6	5.0	2.4	12.2
Fluorescent microagglutination	No.	500	246	254	26	72	18	138
	%	100	49.2	50.8	5.2	14.4	3.6	27.6

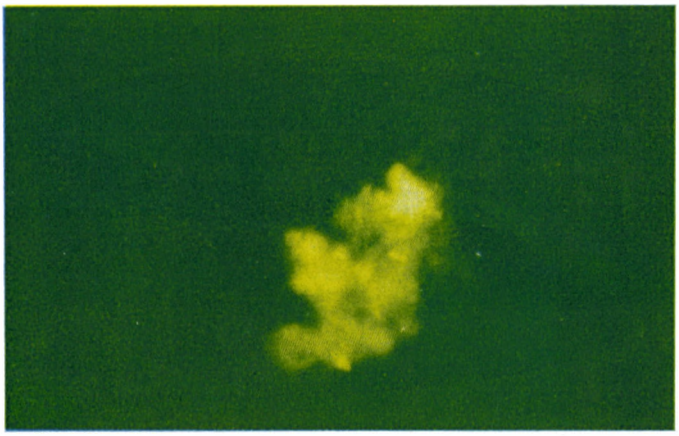

Fig. 1. Microagglutination of *Sh. flexneri*. Magnification, × 250

why the rapid method gave 27.6 per cent more positive results than cultivation, was that with the former technique killed organisms were also detected. Samples giving positive cultures were positive also with microagglutination, as in hospitalized patients dysbiotic conditions usually prevailed, in other words the normal flora was reduced and the pathogenic agent became predominant.

Table II

Comparison of results at cultivation and fluorescent microagglutination during a Sh. flexneri outbreak

Method	Number of samples examined	Positives		Negatives	
		Number	Per cent	Number	Per cent
Cultivation	509	170	33.4	339	66.6
Fluorescent microagglutination	509	301	59.1	208	40.9

Parallel examinations were performed by the two methods during a water-borne *Sh. flexneri* outbreak occurring in a large military unit.

Table II indicates that 509 faecal samples yielded positive results in 33.4 per cent by cultivation and in 59.1 per cent by fluorescent microagglutination. Thus the number of diagnosed cases was 25.7 per cent more when fluorescent microagglutination was employed. One month after the outbreak 6 carriers were found positive with both methods.

Discussion

Fluorescent microagglutination is a rapid diagnostic method characterized by the exactness and sensitivity obtainable by serological methods. It yields results one hour, without cultivation, chiefly in acute enteric diseases.

Certain non-pathogenic enteric organisms are known to share common or related antigens with pathogenic Enterobacteriaceae. The danger of reporting false positive results is lessened to a minimum because, on the one hand, in acute disease a dysbiotic condition prevails and the normal flora generally decreases to numbers unable to cause microagglutination, and, on the other, absorption of *Sh. flexneri* polyvalent serum with *S. typhi* [9, 12] and with S. *typhi murium* [1, 4, 5, 12] decreases the chances of cross-agglutination among pathogenic enteric organisms.

With slight experience amorphous formations giving green autofluorescence can be distinguished from bacterial microagglutination. The former are

never flaky and their edge show a particularly bright fluorescence; in contrast, bacterial aggregates exhibit a fluorescence in the central part. It is important that the Lugol and acridine organe solutions should not be stored for more than 2 or 3 weeks. During longer storage periods acridine orange loses much of its fluorescent staining capacity.

The evaluation of results is easier if several parallel slides are used for each faecal sample. In case of a homologous reaction, the other smears serve as controls: autofluorescing constituents present in the examined sample will appear on all slides.

In order to lessen the danger of infection, it is advisable to add 1 or 2 drops of 10 per cent formalin to all faecal suspensions after distributing them at 2 ml amounts into Wassermann tubes.

We have attempted to compare the sensitivity of our method to cultivation results, to reveal the distribution of identifiable pathogenic agents in patients with enteric disease, and to estimate the possibilities of identifying an organism involved in an outbreak.

It has been shown that fluorescent microagglutination is equally suitable for both kinds of diagnostic work, as the number of positive results exceeded cultivation positivity by a similar percentage.

Immunofluorescent detection is undoubtedly more sensitive than fluorescent microagglutination. However, evaluation of the results with the latter method is less subjective, the examination needs no special fluorochromes and conjugation. Therefore the method can be applied in all hospital laboratories to accelerate the admission of patients to the adequate special wards, to increase the circulation of patients in observation departments, to decrease cross-infections, and to help in prescribing an early aimed treatment.

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GROWTH OF VARICELLA VIRUS IN CONTINUOUS MONKEY KIDNEY AND HUMAN THYROID CELL CULTURES

By

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Summary. The growth of varicella virus has been studied in cultures of HeLa, HEp-2, Detroit-6, and two continuous monkey kidney cell strains (II/1/a, III/1), and in primary cultures of monkey kidney, human thyroid and human embryonic fibroblast cells. Of the cell types tested only the III/1 continuous monkey kidney, the primary monkey kidney, the human thyroid and the human embryonic fibroblast cell cultures were found to support the growth of varicella virus.

A homogenisate of low infectivity was obtained by ultrasonic disintegration of infected cultures of III/1 continuous monkey kidney and of human thyroid cells. The fluid phase of infected, untreated cultures of the same cells exhibited moderate infectivity after low speed centrifugation. The infectivity of the fluid phase somewhat decreased on centrifugation at 300 g, nevertheless it was still present after centrifugation at 12,000 g. Filtration through a G-5 glass filter resulted in complete loss of infectivity.

One of the main problems of varicella virus cultivation is its failure to appear in the fluid phase of tissue cultures. According to WELLER *et al.* [1] this may have the following reasons. (1) Small amounts of infectious virus are produced. (2) The released virions are instable and are inactivated. (3) There is a qualitative difference between the virus replicating in an organism or in a tissue culture system.

Continuing our previous studies in this field [2, 3, 4] we have recently examined some tissue culture systems for their capacity to support the growth of varicella virus. While this work was in progress we have learnt about the studies of CAUNT [5] who has reported on the preparation of infectious virus from homogenisates of primary human thyroid tissue cultures.

Materials and methods

Viruses. Strains isolated and maintained through 80—100 passages in human embryonic fibroblast cultures were used. In certain experiments vesicular contents from varicella patients have served as a direct source of virus.

Preparation of inoculum. Infected fibroblast monolayers were removed from 3 Wassermann tubes by trypsinization with a 0.15 per cent Difco trypsin solution. The cells thus obtained were suspended in a total of 1.8 ml PARKER'S solution. For the infection of tissue monolayers in Wassermann tubes, 0.1 ml of the above suspension was used per tube. Vesicular contents were collected by means of a Pasteur-capillary and used undiluted as inoculum.

Tissue cultures. Continuous cell strains were maintained by weekly transfers in 1 l Roux flasks. From cell strains HeLa, HEp-2, Detroit-6 and from the continuous monkey kidney strains II/1/a, III/1 8×10^4 and 15×10^4 cells were seeded, respectively in 1 ml volume per Wassermann tube each. The monkey cell strains II/1/a and III/1 were obtained from Dr. P. RUZICSKA, Department of Virology, State Institute of Hygiene, Budapest.

Human embryonic fibroblast cultures were prepared from 2 to 3 months old embryos. After removal of the bones, the soft tissues were minced and repeatedly washed in phosphate buffer. The tissue was then trypsinized at 37°C and the cell suspension thus obtained was used for the preparation of Roux-flask cultures. After 1 to 2 weeks incubation the confluent cell monolayer was trypsinized and the cells suspended in nutrient medium so as to obtain 15×10^4 cells/ml. One ml each of this suspension was transferred into Wassermann tubes.

Human thyroid epithelium cultures were obtained from surgical specimens. The thyroid glands were decapsulated, washed in phosphate buffer, and minced. The fragments were pre-treated by three successive trypsinizations at 37°C for 30 minutes each. The final cell suspension was obtained by trypsin treatment at +4°C overnight under continuous magnetic stirring. This was followed by low speed centrifugation in chilled tubes. The sediment was resuspended in nutrient medium. Cultures were prepared in Wassermann tubes with 15×10^4 cells in 1 ml medium per tube.

Preparation of primary monkey kidney cultures was done essentially by the method described by YOUNGNER [9].

Media. HeLa cells were grown in a mixture of 90 per cent PARKER's solution and 10 per cent human serum. For the cultivation of HEp-2, primary monkey kidney and fibroblast cells, 80 per cent PARKER's solution and 20 per cent calf serum was used. The medium for Detroit-6 cultures consisted of 85 per cent PARKER's solution and 15 per cent calf serum. Continuous monkey kidney cell strains (II/1/a and III/1) and human thyroid cells were grown in a mixture of 10 per cent calf serum, 45 per cent HANKS's solution containing 0.5 per cent lactalbumine hydrolysate, and 45 per cent PARKER's medium. The same media were used for growing and maintaining the cultures. The pH was adjusted to 7.2 by 7.5 per cent $NaHCO_3$ solution. All media contained 600 U/ml of penicillin, 400 μg/ml of streptomycin and phenol red as indicator.

Indirect immunofluorescent method. Coverslip cultures were infected with varicella virus. After three days incubation the cells were fixed and treated with an 1 : 10 dilution of Herpes zoster convalescent serum. The preparation was kept in a moist chamber for 30 minutes and then washed. This was followed by a similar treatment with anti-human horse gammaglobulin-bound fluorescein-isothiocyanate. The rinsed preparations were examined by fluorescent microscopy.

Stained preparations. Coverslips measuring 18×18 mm were placed into culture flasks of 8 cm^2 bottom surface and the flask was seeded with cells. After a confluent monolayer had developed the culture was infected with varicella virus. On the third day following the infection the cells were fixed in BOUIN's solution and stained with haematoxylin-eosin.

Disintegration of cells. The cells were disintegrated by ultrasonic vibration in an MSE disintegrator at 1.5 A for 10 minutes.

Results

Cultures of HeLa, HEp-2, Detroit-6, II(1)a and III/1, primary monkey kidney, human thyroid and embryonic fibroblast cells were infected with varicella virus-containing fibroblast cell suspensions or vesicular contents. Of the cell types tested only the monkey strain III/1, the primary monkey kidney, the human thyroid and fibroblast cultures were found to have supported the reproduction of varicella virus.

The cytopathic changes observed in infected primary monkey kidney cultures were identical with those described by WELLER. In the monkey cell line III/1, however, signs of a more perfect virus synthesis were observed. Studies were therefore performed on both the latter cell strain and on primary human thyroid cell cultures in respect to their ability to support varicella virus replication.

Cytopathic phenomena in varicella virus infected cell strain III/1. The first cytopathic change in the native infected cultures manifested itself

on the 5th to 6th day following infection in the form of islands of small, round, refractile cells. The number of such foci increased continuously up to the 10th to 15th day of incubation when the confluent foci formed a network-like pattern. On further progress of the pathologic process part of the infected cells died and the network disintegrated. These cytopathic changes never extended to more than 70 per cent of the monolayer. With occasional fluid changes the cells could be maintained for 3 to 4 months in this state of virus carriership (Fig. 1).

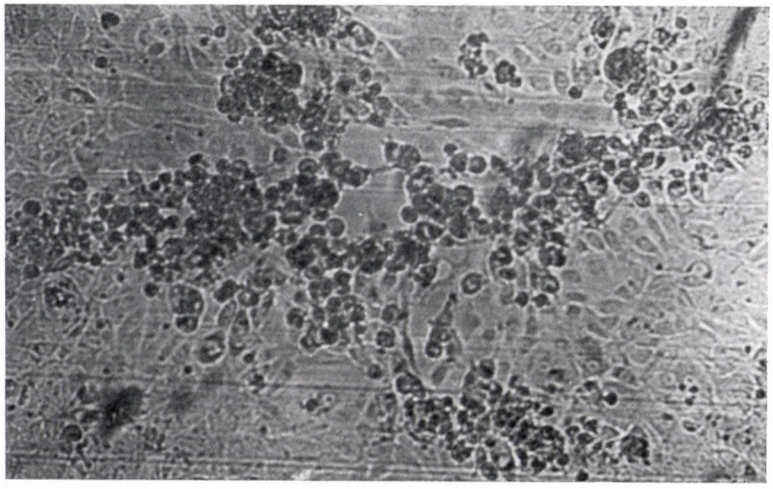

Fig. 1. Cytopathic focus in monkey kidney cell strain III/1 culture infected with varicella virus 10 days earlier. Magnification, about ×300

In coverslip cultures infected with varicella virus and stained with haematoxylin-eosin small groups of cells with eosinophilic nuclear inclusions were demonstrable. In contrast to the development of syncytia in fibroblast cultures no such formations were observed in III/1 cells. The occurrence of cells with 2 to 4 nuclei in the latter cultures was, however, not exceptional (Fig. 2).

Application of the indirect immunofluorescent method revealed the focal presence of cells containing viral material in their nuclei and cytoplasm (Fig. 3).

The cytopathic changes in virus infected human thyroid cell cultures were essentially similar to those observed in cell strain III/1.

The growth of varicella virus in human embryonic fibroblast, continuous monkey kidney and human thyroid cell cultures. Varicella virus-infected fibroblast cell suspension was used as inoculum. A logarithmic dilution series was prepared from the inoculum and 0.1 ml of each dilution was used to infect

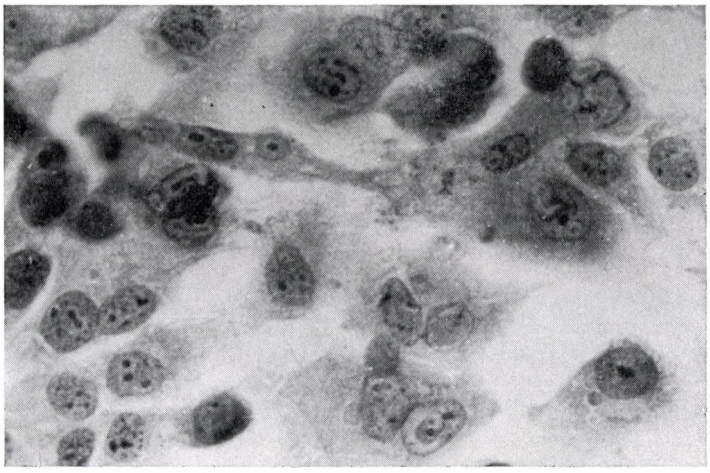

Fig. 2. "A" type intranuclear inclusions in a varicella virus-infected culture of monkey kidney cell strain III/1. Haematoxylin-eosin. Magnification, about $\times 1800$

monolayers of human fibroblast, continuous monkey kidney III/1 and primary human thyroid cells. Infection was performed simultaneously, using the same dilution of virus for each type of cells. Significant differences were observed in virus yields (Fig. 4).

In fibroblast cultures the pathologic foci appeared 24 hours earlier, the rise of titre was more rapid and the final yield higher, than in any other cell type. The titre differences were particularly remarkable (2 log. units) on the second day of the experiment. A tenfold difference persisted until the final

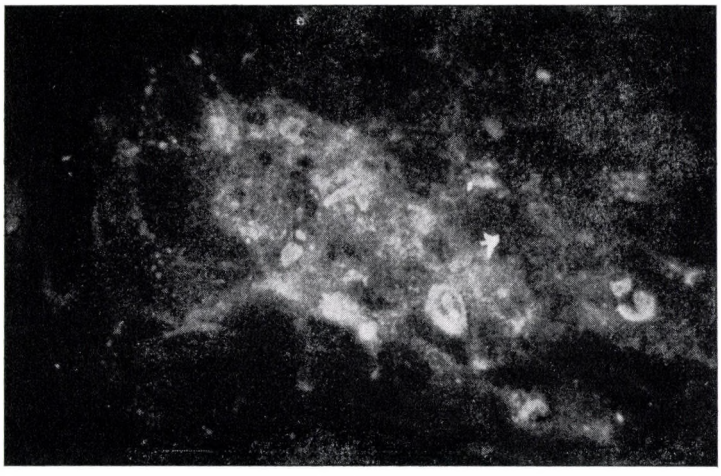

Fig. 3. Specific immunofluorescence in varicella virus-infected culture of monkey kidney cell strain III/1. Magnification, about $\times 1800$

stage. The final yields were identical in both the monkey kidney and the primary human thyroid cells, although the rate of growth was higher in the former cell type up to the 6th day.

In the different cell types infected with identical inocula, the number of foci exhibited an 8 to 10fold difference in favour of the fibroblast cells.

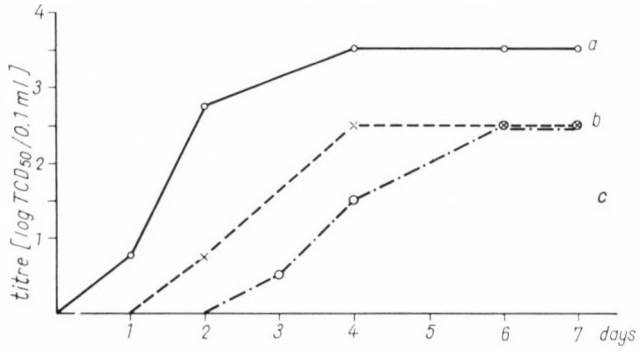

Fig. 4. Growth of varicella-zoster virus in (a) human embryonic fibroblast; (b) monkey kidney cell strain III/1. (c) human thyroid cell cultures

Attempts to obtain cell-free infectious virus in continuous monkey kidney III/1, human thyroid and human embryonic fibroblast cell cultures. Tissue monolayers prepared in Sial flasks of 100 cm² surface were infected with varicella virus. On the 12th day of incubation the fluid phase was removed and centrifuged for 20 minutes at 1300; 5000 and 12,000 *g*. The supernatants were

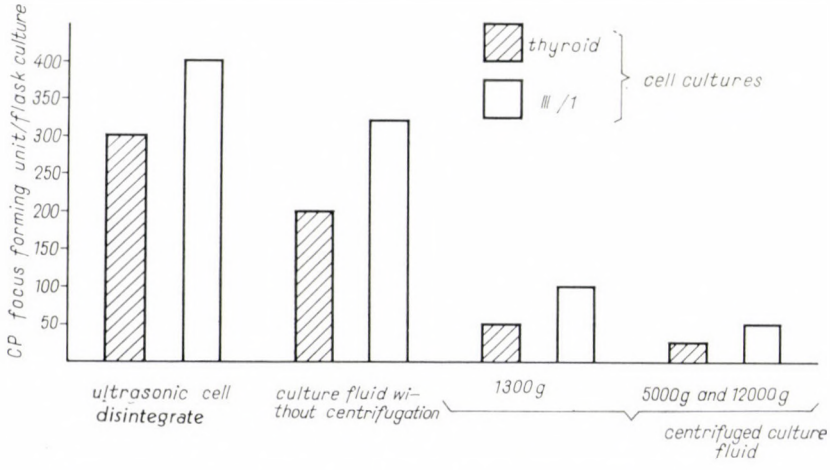

Fig. 5. Infectivity of cell disintegrate and centrifuged culture fluids of varicella virus-infected cell cultures, as measured in human embryonic fibroblast cultures

tested for the presence of virus by inoculating 5 tubes of fibroblast cultures each with 1.0 ml of individual supernatants. The infected cell monolayers (about 8×10^6 cells) were removed by trypsinization, suspended in PARKER's solution and disintegrated by ultrasonic vibration. The material was then centrifuged at low speed and the supernatants used for the infection of fibroblast cultures. The number of foci isolated in fibroblast cultures was calculated for the original volume of fluid or the cellular phase of the corresponding flask culture (Fig. 5).

From the cells of one flask of infected human thyroid or monkey kidney III/1 cell cultures 3 to 4×10^2 focus forming units of infectious virus could be

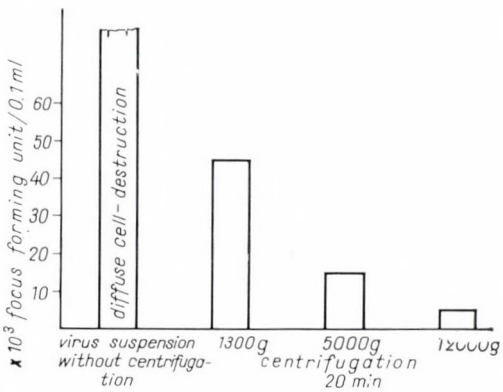

Fig. 6. Infectivity of *Herpes simplex* virus suspensions centrifuged at different speeds, titrated on human embryonic fibroblast cultures

obtained. The infectivity of the fluid phase was, however, remarkably lower. The III/1 monkey cells produced regularly somewhat higher yields of focus forming units. Centrifuged supernatants of varicella virus-infected fibroblast cell cultures were not infective. Supernatants obtained by low speed centrifugation of cell homogenisates of fibroblast cultures contained but 10 focus forming units of virus.

The infectivity of the fluid phase of varicella virus-infected monkey kidney strain III/1 and human thyroid cell cultures exhibited a definite decrease in titre on centrifugation at 5000 or 12,000 g, as compared to that centrifuged at 1300 g. The 12,000 g supernatant failed to contain any cells, thus the titre decrease seemed to have resulted from a sedimentation of part of the virions. This supposition was indirectly supported by another experiment using a cell-free suspension of Herpes simplex virus, where on centrifugation at more than 1300 g for 20 minutes the virus content of the supernatant decreased demonstrably (Fig. 6).

The effect of freezing on the infectivity of cell disintegrates was also studied. Storage for four days at −20°C resulted in a 25fold titre decrease of virus suspensions obtained in monkey kidney III/1 and human thyroid cell cultures.

Discussion

Several cell types cultivated *in vitro* have been studied in respect of their ability to support the growth of varicella virus [1, 7]. On the basis of the number of cytopathic centres and the rate of spread in the culture, human embryonic fibroblast, primary human amnion and HeLa cell types were found to be the most appropriate. Nevertheless, even these cells failed to yield demonstrable amounts of cell-free virus. In contrast to the data of WELLER, WITTON and BELL [1] we did not succeed in infecting the HeLa strain available in our laboratory.

In cultures of the monkey kidney cell strain III/1 the time of appearance and the radial character of spreading of the cytopathic effect was essentially similar to that observed in human amnion and HeLa cell cultures [1, 7]. Generalization of the cytopathic change was, however, never observed in the monkey kidney strain III/1 and 30 to 40 per cent of the cells regularly survived the infection. The formation of syncytia sometimes present in infected amnion cell cultures was not observable in cultures of the continuous monkey kidney cell strain III/1 but occasional cells displayed 2 to 4 nuclei. Comparative experiments have shown that the rate of progression of the cytopathic changes was higher in human fibroblast than in monkey cell strain III/1 or human thyroid cultures.

The phenomena observed in varicella virus-infected monkey cell strain III/1 cultures resembled in many respect those described by CAUNT [5] for the human thyroid cell cultures. The infectivity appearing after ultrasonic disintegration and the characters of infectivity of the culture fluids after centrifugation suggested the production of only moderate amounts of free virus. In cell types used by TAYLOR-ROBINSON [7], WELLER, WITTON and BELL [1] and CAUNT [5] the amount of virus produced might have been even less. In some cases these authors succeeded in liberating very small amounts of virus from human amnion and fibroblast cell cultures. This observation has been supported by our studies on human fibroblast cultures. Cultures of identical counts of fibroblast and monkey strain III/1 or human thyroid cells yielded an average of 10 and 300 to 400 infectious units of virus, respectively. CAUNT succeeded in liberating 2×10^4/ml focus forming units of virus from human thyroid cell cultures. The infectivity of our cell disintegrates was remarkably lower than that. On filtration through glass filter G-5 the infectivity of the homogenisates was lost in our experiments. This might be explained

either by absorption or by inactivation of the virus. The possibility cannot, however, be excluded that the mature virus appearing in the medium is bound firmly to certain cell components.

Chain-like grouping of Herpes simplex virions was observed in electron-microscopic preparations by SMITH [8]. This fact may be one of the explanations for the partial sedimentation of herpes and varicella viruses on centrifugation at 5000 or 12,000 g.

The titre decrease of ultrasonically disintegrated suspensions of infected human thyroid or monkey kidney III/1 cell cultures observed on storage at —20 °C is in agreement with the observations of CAUNT [5] who found a similar decrease of infectivity in disintegrates stored at —63 °C.

The encouraging observations on the growth of varicella virus in monkey kidney strain III/1 and human thyroid cell cultures suggest the possibility that by further improvements in the conditions of cultivation these systems may become valuable tools in the production of large amounts of varicella virus.

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COLUMN CHROMATOPGRAPHY ON DEAE CELLULOSE COLUMN OF HERPES SIMPLEX VIRUS AND CYTOMEGALOVIRUS

By

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Summary. The elution of Herpes simplex virus and Cytomegalovirus from DEAE cellulose columns was studied using phosphate buffers with different sodium chloride contents as eluents.

Herpes simplex virus and Cytomegalovirus were eluted by 0.6 or 0.9 *M* and by 0.3 *M* sodium chloride in phosphate buffer, respectively.

The bulk of cellular proteins was eluted by 0.3 *M* sodium chloride in phosphate buffer thus only the Herpes simplex virus was partially purified by this method.

The elution of complement fixing activity and infectivity of both viruses ran parallel in each fraction.

A number of reports has recently been published on the use of column chromatography for purification of viruses [1, 2], isolation of different virus-induced products [3, 4], and for the separation of different virus mutants [5, 6, 7] or strains [8, 9, 10]. In the present study the chromatographic purification of Herpes simplex virus has been attempted and the conditions for optimal elution from DEAE cellulose columns of Herpes- and Cytomegaloviruses were examined. The two viruses under study are members of the same group and the possible differences in their surface characters were supposed to result in different elution requirements.

Materials and methods

Viruses. The strain of Herpes simplex virus used in the present study had been isolated in this laboratory in 1962. Since that time the virus has been maintained by serial transfers. The cytopathogenic effect of the strain is of the mixed type, *i.e.* both small groups of round cells and polykaryocytes are detectable in the infected cultures. This character is similar to that described by Hoggan and Roizman [11] for their Herpes simplex strain isolated in 1959. The mixed character of the cytopathogenic effect was a result of the simultaneous presence of two mutants in our Herpes simplex virus. The individual mutants were successfully separated by the end point dilution method.

The Cytomegalovirus strain was kindly supplied by Dr. H. Stern (St. George's Hospital Medical School, London) and then maintained in our laboratory by serial passages in human embryonic fibroblast cell cultures.

Tissue cultures. For growing and infectivity titration of Herpes simplex virus and of Cytomegalovirus, HeLa and human embryonic fibroblast cell cultures, respectively, were used. HeLa cells were cultivated in Parker's No. 199 medium with 10 per cent human serum, while the medium for human embryonic fibroblast cultures consisted of Hanks' solution containing 0.5 per cent lactalbumine hydrolyzate and 20 per cent bovine serum. For growing and titrating viruses both types of cultures were maintained in Parker's No. 199 medium without serum (Herpes simplex virus) or with 20 per cent bovine serum (Cytomegalovirus).

Immune sera. Anti-Herpes simplex sera were obtained from rabbits inoculated with the virus by the corneal scarification method and bled 3 weeks after the development of keratitis.

Sera against Cytomegalovirus were obtained from patients exhibiting specific complement-fixing antibody titers of 1 to 128 or higher.

DEAE cellulose. A standard lot of N-diethylaminoethyl (DEAE) cellulose was used after preparation according to PETERSON and SOBER [12].

Chromatography. After appropriate preparation, 1.4 g samples of DEAE cellulose were made up into columns of 1×10 cm each. The columns were equilibrated with 0.02 *M* phosphate buffer of pH 7.1.

Virus suspensions for chromatography were obtained by suspending the infected cells in the fluid phase of the culture and homogenizing the suspension in an MSE type ultrasonic apparatus (1.5 A, 60 sec., 10 ml suspension, $^3/_4''$ titanium head). Debris were eliminated by centrifugation and the supernatant was dialyzed for 24 hr. against 0.02 *M* phosphate buffer of pH 7.1. Of the material thus obtained 5 ml samples were pipetted onto the chromatographic columns. The eluents used consisted of a 0.02 *M* phosphate buffer of pH 7.1 alone or with 0.3, 0.6 or 0.9 *M* sodium chloride content. Elution was accomplished by collecting four 5 ml samples with each individual eluent. Perfusion was enhanced by applying 200 water cm pressure. Under such conditions the perfusion rate was 0.5 ml/min.

Examination of the fractions. The protein content of the fractions was expressed in extinction values at 280 mμ wawelength as measured in the spectrophotometer. The TCID 50 of the Herpes simplex virus and Cytomegalovirus was determined according to REED and MUENCH [13] by titration in HeLa and in human embryonic fibroblast cell cultures, respectively. Subsequently the fractions obtained with the individual eluents of the same sodium chloride molarity were pooled. The pools were concentrated tenfold by dialysis against powdered dextrane.The concentrates were dialyzed against saline overnight and their complement-fixing antigen content was determined by TAKÁTSY's micromethod [14].

Results

Results obtained with Herpes simplex virus are presented in Fig. 1. Fig. 1/a shows the protein content (extinction at 280 mμ) and the infectivity of the individual fractions, while Fig. 1/b the complement-fixing antigen content of the same fractions.

The bulk of cellular proteins and some minute quantity of Herpes simplex virus were eluted by the eluent containing 0.3 *M* sodium chloride. Maximum elution of Herpes simplex virus was obtained with eluents containing 0.6 or 0.9 *M* sodium chloride. This method failed to separate the two mutants characterized by different types of cytopathogenicity.

In the complement fixation experiments the titre of the antigen was determined as the highest antigen dilution yielding maximum titres with a standard dilution series of a specific Herpes simplex virus immune serum. As shown in Fig. 1/b, elution of the complement-fixing activity and of infectivity was parallel.

The same experiment was carried out also with Cytomegalovirus. The results are presented in Fig. 2.

The bulk of infectivity was eluted by a phosphate buffer of 0.3 *M* sodium chloride content. At concentrations up to 0.9 *M* the amount of eluable infectivity decreased. Elution of complement-fixing activity ran essentially parallel with that of infectivity.

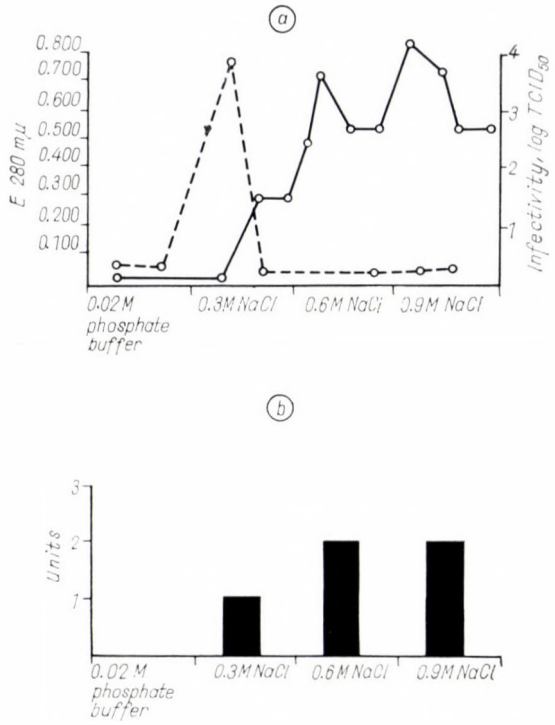

Fig. 1. Chromatography of Herpes simplex virus on DEAE cellulose column
a) proteins from Herpes simplex virus- infected cells: ○- - - -○, infectivity:○————○
b) elution of complement-fixing antigen

An attempt was made to recover separately the Herpes simplex virus and the Cytomegalovirus from mixtures of equal amounts of sonically disintegrated and dialyzed cultures of the individual viruses. Elution was performed as described above. Titration of the individual fractions was carried out in parallel on HeLa and fibroblast cultures. As the sensitivity of these cells is selective to cytopathogenic effects of the two viruses under study, this served as further means of differentiation. Results obtained in this type of experiment are presented in Fig. 3.

Discussion

Chromatography on DEAE cellulose was found to be a useful method for the partial purification of Herpes simplex virus. Parallel to a remarkable decrease of the total protein content of infected tissue culture homogenisates, a nearly 100 per cent recovery of infectivity was achieved. Cytomegalovirus could also be recovered after chromatography but in this case no purification

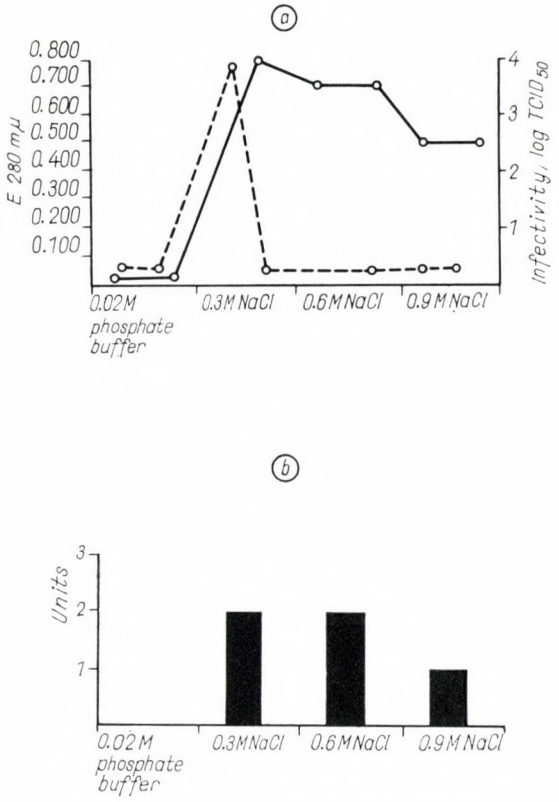

Fig. 2. Chromatography of Cytomegalovirus on DEAE cellulose column
a) proteins from Cytomegalovirus infected cells: ○-----○
infectivity: ○ ——— ○ *b)* elution of complement-fixing antigen

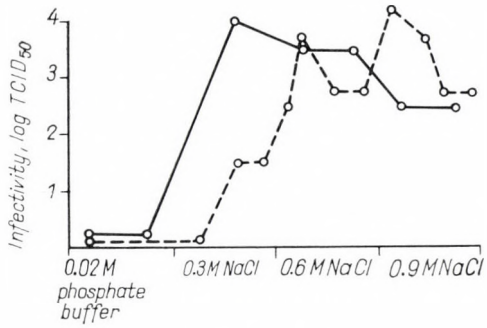

Fig. 3. Separation of Herpes simplex virus and Cytomegalovirus by chromatography on DAEA cellulose column. Herpes simplex virus infectivity: ○ ------ ○
Cytomegalovirus infectivity: ○ ——— ○

was possible as both the infectivity and the bulk of total protein content was eluted by a phosphate buffer containing 0.3 *M* sodium chloride. Elution of the complement-fixing antigen and the infectivity of both viruses ran completely parallel. Thus under the experimental conditions used we were unable to demonstrate the presence of a separate complement-fixing antigen described by RUSSEL *et al.* [15] for Herpes simplex virus.

Attempts to separate the two mutants of Herpes simplex virus by chromatography has failed in our system. This is in contrast with the findings of ROIZMAN [7] and KOHLHAGE [16] who succeeded in separating the two mutants by chromatography on calcium phosphate and Ecteola cellulose columns, respectively. The reason for our failure might have been the lability of the mutants, resulting in rapid reversion on the first passage in tissue culture.

It seemed interesting that the conditions for the elution of Herpes simplex virus and of Cytomegalovirus from DEAE cellulose columns were remarkably different. However, this difference was not due to the use of different cell types for cultivating the two agents. The chromatographic characters of Herpes simplex virus grown in fibroblast cell cultures were identical with those described above for the same virus grown on HeLa cells. The difference in the conditions of elution of Herpes simplex virus and Cytomegalovirus seems to have been caused by some structural peculiarities of the respective virions. Such differences between viruses belonging to the same group have been described. HARUNA *et al.* [8] reported on similar findings for adenoviruses, KOZA [10] and THOMSSEN and MAASS [17] for avirulent strains of poliovirus and SIMON and DÖMÖK [6] for the haemagglutinating and non-haemagglutinating mutants of echoviruses. With these findings in mind we only conclude that the conditions for the elution from DEAE cellulose columns of Herpes simplex virus and Cytomegalovirus are different. The exact causes of the phenomenon are not yet known.

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FATTY ACIDS IN ENTERIC BACTERIA

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Summary. (i) The fatty acid spectrum of *Escherichia coli*, *Salmonella typhi-murium*, *Shigella flexneri* and *Proteus vulgaris* has been determined. The organisms contained identical fatty acids but differed in the quantitative distribution of these substances.

(ii) In the examined bacteria fatty acids containing 16 carbon atoms predominated.

(iii) As compared to the other strains, in *Sh. flexneri* and *P. vulgaris* cyclopropane ring-containing fatty acids occurred in a considerably higher proportion.

(iv) It has been concluded that the fatty acid spectrum cannot be used for the differentiation of closely related bacterial species.

The lipid composition of bacteria has scarcely been examined. Development of analytical lipid research has made it possible to elucidate the distribution and metabolic role of these substances in various organisms. The present paper deals with the fatty acid spectrum in some enteric bacteria and its changes during cultivation. We have attempted to show whether bacterial species can be differentiated or identified on the basis of fatty acid determinations.

Materials and methods

Cultures. Escherichia coli 30006*, *Salmonella typhi-murium*, *Shigella flexneri*, *Proteus vulgaris* 61001.*

Culture medium. Lipid-free casein hydrolyzate (Difco), 10 g; sodium chloride, 5 g; disodium hydrophosphate, 5 g; ferrous ammonium sulphate, 0.03 g; magnesium sulphate, 0.01 g; manganese sulphate, 0.01 g; distilled water, to make 1 litre solution.The pH was adjusted with sodium hydroxide to 7.2 Only analytical grade reagents were used.

The bacteria were grown in 500 ml Erlenmeyer flasks containing 150 ml medium. The flasks were immersed in a water bath and shaken at a rate of 120 cycles per minute. The temperature of incubation was $37 \pm 0.1°C$; 8 and 18 hour cultures were used.

Reagents. Apieson L, polyethylene glycoladipate and Celit 545 (100—120 mesh) (Applied Science Lab., State College, Pa., U. S. A.); analytical grade potassium hydroxide, analytical grade hydrochloric acid distilled from stannous chloride; analytical grade methanol and diethyl-ether distilled from ferrous sulphate; anaıytical grade acetone; nitrosomethylurea.

Preparation of bacteria for fatty acid analysis. The cultures were separated from the medium and washed in saline by centrifugation at 2800 r. p. m. The washed cells were suspended in acetone and stored for 22 hours at 4°C. After filtration through G4 glass filter, the bacteria were dried to constant weight in a vacuum exsiccator over phosphorus pentoxide.

Extraction of fatty acids. One g of dried cells was refluxed for 4 hours over boiling water in 15 ml 20 per cent potassium hydroxide dissolved in a mixture containing equal volumes of methanol and water. After cooling to room temperature, the solution was acidified with H_2O_2-

* Registration numbers Culture Collection Centre, State Institute of Hygiene, Budapest.

free hydrochloric acid diluted 1 : 3. The solution was then shaken 3 times with 15 ml aliquots of diethyl ether. The ether extracts were united and shaken out 3 or 4 times with 5 per cent aqueous potassium hydroxide until the alkali became colourless. Acidifying and shaking with ether were repeated and the united ether extract was dried over a 4 : 1 mixture of sodium sulphate and sodium hydrocarbonate. Then the extract was evaporated in inert (CO_2 or N_2) atmosphere at 40°C and dried in the vacuum exsiccator over phosphorus pentoxide until constant weight had been reached.

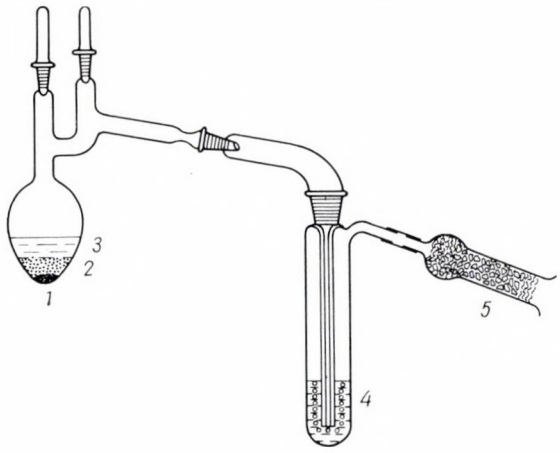

Fig. 1. Glass apparatus used for conversion of fatty acids into methyl esters. 1 = nitrosomethylurea; 2 = 50 per cent potassium hyroxide; 3 = diethylether; 4 = fatty acid sample dissolved in diethylether; 5 = pumice impregnated with 25 per cent acetic acid

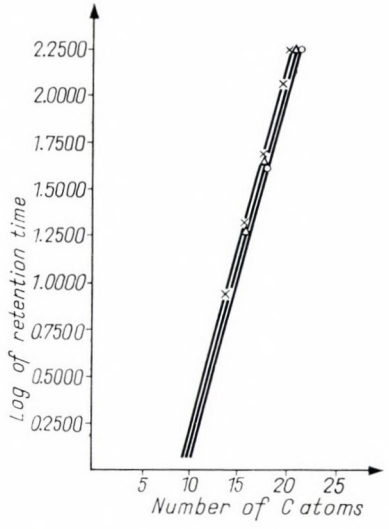

Fig. 2. Standard graph for fatty acid series. Pye Argon chromatograph; Celit 545 + 10 per cent PEGA column; gas flow, 40 ml/min; 178°C. × = saturated fatty acids, △ = fatty acids with 1 unsaturated bond, ○ = fatty acids with 2 unsaturated bonds

Preparation of fatty acids for gas chromatography. Conversion of fatty acids to methyl esters was performed by the method of AMSTUTZ and MYERS [1] in the apparatus shown in Fig. 1. The diazomethane reagent was prepared freshly from nitrosomethylurea. Ether solution of the obtained fatty acid methyl esters were evaporated in inert atmosphere then placed in the vacuum exsiccator over phosphorus pentoxide and dried to constant weight.

Gas chromatography. The W. G. Pye Argon chromatograph supplied with a beta ray ionization detector containing 20 μC Sr^{90} was used. Argon (99.95 per cent purity) passed through a 5Å molecular filter was employed as carrier gas.

By the use of 121.92 cm long borosilicate glass columns two charges of different polarity were prepared: (a) Celit 545 with 10 per cent Apieson L; (b) Celit 545 with 10 per cent polyethylene glycol adipate. Detector current, 1250 V; travelling speed of recording paper, 15.24 cm hour.

Concentrated fatty acid methyl esters were placed at 0.1 μl aliquots on the columns with a Pye magnetic pipette and an auxiliary device constructed in our laboratory.

Identification of fatty acids was checked by methyl ester preparations of myristic, palmitic, stearic, arachic, oleic, erucic, palmitoleic, linolic and linolenic acid.

In order to identify unsaturated components, one preparation of the examined samples was gas chromatographed before and after catalytic hydrogenization carried out as described by POUKKA *et al.* [2].

Fatty acid components of the bacteria were identified by the use of the above standard preparations. Graphs for homologous fatty acid series with saturated and 1 and 2 unsaturated bonds were drawn so that the abscissa represented the number of carbon atoms and the ordinate the logarithm of the retention time (Fig. 2.)

Results

Figs 3 and 4 show the fatty acid spectrum determined in 8 and 18 hour shaken cultures of four different bacteria. It is seen that abundant amounts of C_{10}—C_{20} fatty acids were demonstrated. This finding indicates the important role of these substances in the metabolism of the examined organisms. The significant difference between the fatty acid spectrum of 8 and 18 hour cultures also supports this consideration. In *E. coli*, *S. thyphi-murium* and *Sh. flexneri* qualitatively similar, but quantitatively varying spectra were demonstrated. The fatty acid spectrum of *P. vulgaris*, however differed considerably from that of the above bacteria.

The percentage distribution of fatty acids calculated from the graphs shown in Figs 3 and 4, is presented in Table I. In the examined bacteria 8 or 9 different C_{10}—C_{20} fatty acids were present. Three substances predominated, the others occurred in amounts of less than 10 per cent.

For the sake of simplicity, bacterial fatty acids have not been listed separately. In Table II these compounds are grouped as components containing less than 16, 16 and more than 16 carbon atoms. The main components were C_{16} fatty acids. In *Sh. flexneri* and *P. vulgaris*, however, higher fatty acids occurred in considerable amounts (25—48 per cent). In contrast, *E. coli*, and *S. typhi-murium* contained only in 9—12 per cent of these substances. The difference is remarkable, although the significance of these fatty acids in pathogenicity and resistance is as yet unknown.

The former bacteria were also characterized by a higher content of cyclopropane ring fatty acids (*P. vulgaris*, 35 per cent, *Sh. flexneri*, 24 per cent).

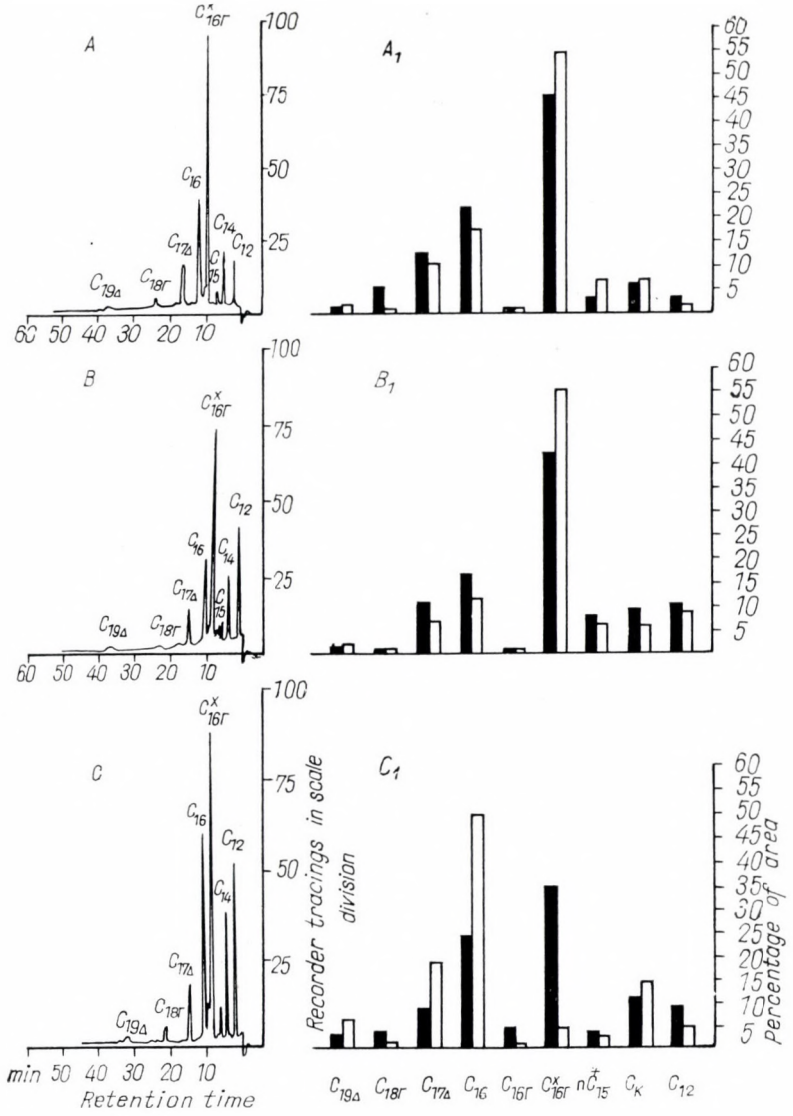

Fig. 3. Fatty acids in enteric bacteria. ■ = 8 hour culture, □ = 18 hour culture; A, A_1 = = *E. coli;* B, B_1 = *S. typhi-murium;* C, C_1 = *Sh. flexneri*

Table I

Fatty acid composition of enteric bacteria

No.	Fatty acids	Designation	8 hour cultures				18 hour cultures			
			1	2	3	4	1	2	3	4
1	Hexadecanoic acid*	$C_{16:1}$*	46	42	35	6	54	55	4	3
2	n-Hexadecanoic acid (palmitic acid)	C_{16}	22	17	24	48	18	12	49	44
3	Methylene hexadecanoic acid	$C_{17}\triangle$	13	11	8	16	10	7	19	21
4	n-Tetradecanoic acid (myristic acid)	C_{14}	6	9	11	13	7	6	15	15
5	n-Dodecanoic acid	C_{12}	3	11	9	+	2	9	5	+
6	Methylene oxadecanoic acid	$C_{19}\triangle$	2	2	2	13	2	2	6	15
7	n-, iso- and aiso-Pentadecanoic acids	n, i, ai-C_{15}	3	8	3	3	7	7	2	2
8	Hexadecenoic acid (palmitoleic acid)	$C_{16:1}$	+	+	4	+	—	+	+	+

Data are expressed in per cent of total area. Organisms: 1 = *E. coli*, 2 = *S. typhi murium*, 3 = *Sh. flexneri*, 4 = *P. vulgaris*.

* Proved to be identical with beta hydroxymyristic acid.

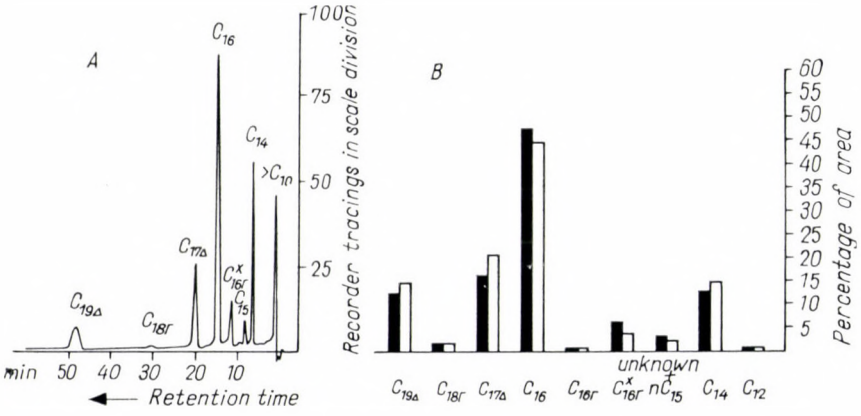

Fig. 4. Fatty acids in *P. vulgaris* ■ = 8 hour culture, □ = 18 hour culture

Table II

Percentage distribution of fatty acids in 18 hour cultures of enteric bacteria

Organism	Fatty acid with less then 16 C atoms	Fatty acids with 16 C atoms	Fatty acids with more than 16 C atoms	
			Total	Fatty acids with cyclo-propane ring
E. coli	22	72	12	12
S. typhi-murium	22	71	9	9
Sh. flexneri	22	54	25	24
P. vulgaris	17	48	37	35

Discussion

Identification of bacteria on the basis of chemical composition has long been attempted. In some organisms (*e. g.* salmonellae) various types may be distinguished within the species by determining certain special carbohydrates. In view of the high sensitivity of gas chromatography, attempts have been made to use the fatty acid spectrum for differentiation purposes. Examining the fatty acid composition of *C. diphtheriae*, *C. ovis*, *P. pestis* and *B. subtilis*, ASSELINEAU [3] showed that every species possessed a characteristic fatty acid spectrum; the differences within the species were not so definite. ABEL *et al.* [4], who investigated fatty acids in various members of the order Eubacteriales, concluded that the method may be useful for the identification of bacteria.

The present examinations revealed that the four Enterobacteriaceae strains *(E. coli, S. typhi-murium, Sh. flexneri* and *P. vulgaris)* contained identical fatty acid components. Although these species differed in the quantitative distribution of the components, our findings indicate that determination of the fatty acid spectrum was insufficient for identification purposes. It is important to consider the fact that the lipid composition and consequently the fatty acid spectrum highly varies with culturing conditions such as temperature, pH and ingredients of the medium. This explains discrepancies between the results of some authors *(e.g.* between those of ABEL *et al.* [4] and MARR and INGRAHAM [5]). The high variability makes it improbable that the fatty acid spectrum could be relied upon for the differentiation of closely related species. On the other hand, the method may be adequately applied in the case of unrelated species.

From the present findings it is clear that in *E. coli*, *S. typhi-murium* and *Sh. flexneri* the main component is a C_{16} fatty acid containing presumably 2 unsaturated bonds. This substance is not identical with palmitoleic acid.

Its unsaturated bond content has not been confirmed. The intermediary role in fatty acid metabolism of the unidentified component is indicated by the finding that its proportion varies with the time of incubation: it considerably decreases in 18 hour *Sh. flexneri* cultures. At the same time the amount of cyclopropane ring-containing higher fatty acids increases. The unidentified substance (which now has proved to be identical with beta hydroxymyristic acid) may serve as a precursor in the synthesis of cyclopropane ring fatty acids. In *E. coli*, *S. typhi-murium* and *Sh. flexneri* palmitic acid comprised the second most important lipid component.

The fatty acid spectrum in *P. vulgaris* somewhat differed from that found in the above three organisms. In *P. vulgaris*, in addition to palmitic acid, cyclopropane ring-containing methylene hexadecanoic, methylene octadecanoic and myristic acids occurred in considerable amounts.

Cyclopropane ring-containing methylene hexadecanoic acid was common especially in *Sh. flexneri* and *P. vulgaris*. This fatty acid was first detected by means of radioactive tracing by O'LEARY [6]. Later it was isolated by DAUCHY and ASSELINEAU [7]. The cyclopropane ring structure was demonstrated by KANESHIRO and MARR [8], who showed the presence of a cis-9-12 methylene bridge. Their finding was confirmed by CHALK and KODIČEK [9].

Little is known about the biological role of these special fatty acids and about the association between their presence and the biological properties of bacteria. Working with *Staphylococcus aureus* strains differing in antibiotic sensitivity, we have shown that with the broadening of the resistance spectrum there is an increase in the proportion of cyclopropane ring fatty acids [10]. According to HOFMANN and PANOS [11], fatty acids containing long chain cyclopropane ring enhance the multiplication of bacteria, may in some respect supplement biotin, and act as metabolic activators. Thus, their increased occurrence indicates a more active cellular metabolism.

From the present experiments it may be concluded that, although the determination of the fatty acid spectrum does not seem suitable for identification and systematic purposes, it is desirable to elucidate the biosynthesis of special fatty acids and their influence on the biological properties of the cell.

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STUDIES ON LIPIDS IN PSEUDOMONAS PYOCYANEA

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Summary. The phosphatide and fatty acid composition of a *Pseudomonas pyocyanea* strain grown for various times, has been investigated. The following results were obtained.

(i) The organism contained 7.2—7.7 per cent total pure lipid, of which 75—80 per cent consisted of phosphatides.

(ii) The total phosphatide content in dried bacteria increased with the culture's age.

(iii) Phosphatides were composed mainly of lecithin and cephalin-type compounds.

(iv) As revealed by thin layer chromatography, phosphatides contained 5 components. The same components were found in 3 cultures of different ages.

(v) Three cultures of different ages contained uniformly 12 fatty acids.

(vi) The main fatty acid components was palmitic acid. Fatty acids containing more than 16 carbon atoms comprised 90 per cent of the total fatty acid content.

(vii) In older cultures fatty acids with C_{19} cyclopropane ring were prevalent.

Phosphatides play an important part in cellular function. As constituents of the cell membrane they exert a particular influence on the entry into the cell of various substances and on metabolic processes. In spite of this, little is known about the constitution of phosphatides. However, certain recently developed microanalytical methods have allowed some progress in the elucidation of their nature.

As reported in previous papers [1, 2], an association has been revealed between the lipid content and antibiotic sensitivity of bacteria. The present paper describes further studies on this problem, in which thin layer and gas chromatography experiments have been performed for the determination of the phosphatide and fatty acid contents of a *Pseudomonas pyocyanea* strain isolated in our institute.

Materials and methods

Strain. The experiments were carried out with 8, 18 and 48 hour cultures of the same *Ps. pyocyanea* strain. The strain was sensitive to streptomycin, chloramphenicol, erythtromycin and polymyxin, and resistant to penicillin and tetracycline.

Medium. Lipid-free casein hydrolyzate (Difco), 10 g; sodium chloride, 5 g; disodium hydrophosphate, 5 g; potassium dihydrophosphate, 5 g; ferrous ammonium sulphate, 0.03 g; magnesium sulphate, 0.01 g; manganese sulphate, 0.01 g; distilled water to give 1000 ml solution. The pH was adjusted to 7.2. Only analytical grade reagents were used.

Cultivation was carried out in 500 ml Erlenmeyer flasks containing 150 ml medium. The flasks were immersed in a water bath of $37 \pm 0.1°C$ and shaken at a rate of 120 cycles per minute. Incubation lasted for 8, 18 and 48 hours.

Apparatuses. (A) The apparatus (to be published; applied for patent) devised by the authors for thin layer chromatography allowed the preparation of layers of required thickness.

(B) Pye Argon gas chromatograph supplied with electronic integrator.

Reagents. (a) Thin layer chromatography: Analytical grade acetone, chloroform, methanol, ethanol, ethylether (distilled from ferrous sulphate) (Reanal, Budapest); phosphomolybdic acid, Silicagel G (Merck, Darmstadt); Whatman's cellulose powder.

(b) Gas chromatography: Celit 545 (100—120 mesh), polyethylene glycoladipate (PEGA). Apieson L (APL) (Applied Science Laboratory, State College. P. a., U. S. A.); argon gas, 99.95 per cent purity (Nitrogen Works, Pét), analytical grade potassium hydroxide and hydrochloric acid (distilled from stannous chloride).

Nitrosomethylurea was prepared in this institute.

I. Thin layer chromatography

(1) *Preparation of layers :* 6×20 cm mirror glass plates, 3 mm thick; layer thickness 500 μ; activation, 10—15 minutes at room temperature then 1 hour at 110°C.

(2) *Solvent :* chloroform: methanol: water (65 : 25 : 4) as described by WAGNER and HÖRHAMMER [3]. Chromatograms were run for 50—60 minutes by the ascending technique at room temperature.

(3) *Detection of spots :* (a) General lipid reagent: 5 per cent phosphomolybdic acid in ethanol [4].

(b) Detection of lecithin-type components: DRAGENDORFF's reagent [5].

(c) Detection of amino group-containing phosphatides: ninhydrin reagent as modified by MOFFAT and LYTTLE [6].

(d) Detection of plasmalogens: SCHIFF's reagent [7].

(e) Detection of phosphatides containing unsaturated bonds: iodine vapour as described by MANGOLD [4].

(4) *Preparation of bacteria for phosphatide analysis.* After incubation the bacteria were separated from the medium by centrifugation at 2800 r.p.m. then washed in saline. The washed bacteria were kept in acetone for 22 hours at 4°C. After filtration through G4 glass filter, the bacteria were dried to constant weight over phosphorus pentoxide.

(5) *Extraction of bacteria.* Floor-fine ground dried bacteria, 1.5 to 2 g in weight, were extracted in a modified Soxhlet apparatus with 140 ml chloroform-methanol mixture (2 : 1) for 4 hours in 3 fractions (60 ml, 2 hours; 40 ml, 1 hour; 40 ml 1 hour). The united fractions were then evaporated to dryness under reduced pressure and CO_2 flow at 45°C, in a weighed test tube. Finally the extract was dried to constant weight over phosphorus pentoxide in a vacuum exsiccator.

Because of its free amino acid and lipopolysaccharide content, the "crude" lipid preparation obtained in this manner was subjected to further purification.

(6) *Purification of the crude lipid preparation :* Of various purification procedures the method of LEA and RHODES [8] yielded the most satisfactory results. Column chromatography was carried out through a 40×2 cm column containing 22 g of Whatman's cellulose powder. Elution was performed with a chloroform—ethanol (4 : 1) mixture. Five ml fractions were chromatographed for 18 hours on Schleicher—Schuell 2045b Mgl paper by the unidimensional ascending technique. The solvent was tetrahydrofuran: ether: water (60 : 15 : 10). The first 35 ml of eluate was discarded as it contained no phosphatides. The further 100 ml fraction (pure lipid preparation) was then evaporated to dryness and dried to constant weight.

(7) *Separation into acetone-soluble (fatty acid) and into acetone-insoluble (phosphatide) fractions* was carried out by LEUTHARDT's method [9]. The pure lipid preparation was dissolved in ether, then 0°C acetone was added (approx. 1 : 3). Acetone caused the precipitation of phosphatides, but left the free fatty acids and neutral lipids in solution. The mixture was kept at 4°C for 24 hours. Lipids were then separated from the acetone-soluble fraction by passing through G4 glass filter.

(8) *Separation of phosphatides into lecithin and cephalin.* The phosphatides were redissolved in ether, then cephalin was precipitated with absolute ethanol. After standing in the refrigerator at 4°C for 24 hours, the precipitate was separated from the ethanol-soluble lecithin by means of a G4 glass filter. Drying to constant weight was performed over phosphorus pentoxide in a vacuum exsiccator.

II. Gas chromatography

(1) *Preparation of bacteria for fatty acid analysis.* Centrifugation, washing and drying of bacteria were performed as described under I/4.

(2) *Extraction of fatty acids:* Dried bacteria weighing 1 g were heated under a reflux for 4 hours in 15 ml 20 per cent potassium hydroxide dissolved in a mixture containing equal

volumes of water and methanol. After cooling to room temperature, fatty acids were liberated with 1 : 3 hydrochloric acid (analytical grade), then shaken out 3 times with ether. The pooled ether extracts were dried over sodium sulphate and sodium hydrocarbonate (4 : 1). After filtration the extract was evaporated at 40°C, then dried over phosphorus pentoxide to constant weight.

(3) *Conversion of fatty acids to methyl esters* was carried out with diazomethane prepared freshly from nitrosomethylurea as described by AMSTUTZ and MYERS [10]. The obtained methyl esters were gas chromatographed.

Results

Results of thin layer chromatography. The lipid content of *Ps. pyocyanea* cultures of various ages is presented in Table I. It is clear that the cultures did not differ in total lipid content. The percentage data for total pure lipid are interesting, as they show the degree of contamination of the crude preparations. The crude lipid content was 9—10 per cent more than the pure lipid content. The difference is due to the presence of free amino acids and a lipopolysaccharide-protein complex in the crude preparation. The phosphatide content varied

Table I

Lipid content of Ps. pyocyanea cultures of various ages

Age of culture, hours	Percentage values for dried bacteria*		
	Total crude lipid	Total pure lipid	Phosphatide
8	8.0	7.2	5.0
18	7.9	7.2	5.5
48	8.6	7.7	6.0

* Data indicate average values obtained in two parallel cultivations

considerably with the age of the culture. The 8 hour culture contained 5 per cent, while the 48 hour culture 6 per cent, phosphatide. Bacteria incubated for 18 hours occupied an intermediary position (5.5 per cent).

In further experiments the phosphatide components were examined by thin layer chromatography. The results are shown in part (a) of Fig. 1. Chromatography was carried out by the ascending technique on a 500 μ thick Silicagel G layer using chloroform: methanol: water (65 : 25: 4) solvent. Spots were detected by spraying with 5 per cent phosphomolybdic acid dissolved in ethanol and with concentrated sulphuric acid. Part (b) of Fig. 1 indicates ninhydrin positive spots.

Fig. 1 indicates that the three cultures of various ages contained 5, qualitatively identical phosphatide components.

Spot 1' reacted only with ninhydrin and was therefore regarded as an amino acid contamination.

Spot 1 gave positive colour reaction with phosphomolybdic acid, iodine, ninhydrin and also with SCHIFF's reagent, and therefore corresponded to acetalphosphatide (plasmalogen).

Spot 2 was lysocholamine cephalin, as it reacted with phosphomolybdic acid, iodine and ninhydrin.

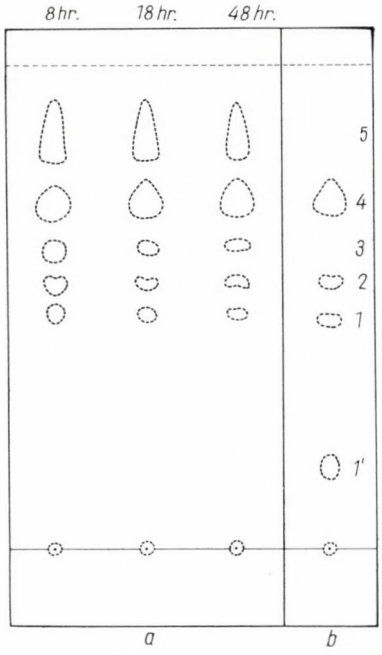

Fig. 1. Thin layer chromatogram of phosphatides in 8, 18 and 48 hour *Ps. pyocyanea* cultures. Silicagel G layer, 500 μ; chloroform: methanol: water solvent (65 : 25 : 4); detection: (a) phosphomolybdic acid + concentrated sulphuric acid, (b) ninhydrin. Components: 1' = amino acid; 1 = plasmalogen, 2 = lysocholamine cephalin, 3 = lecithin, 4 = cholamine cephalin, 5 = cardiolipin

Spot 3 corresponded to lecithin, as it reacted with phosphomolybdic acid, iodine and DRAGENDORFF's reagent.

Spot 4 was the most intensive component on the chromatogram. As it gave colour reaction with phosphomolybdic acid, iodine and ninhydrin, it was regarded as cholamine cephalin.

Spot 5 bound strongly Rhodamine 6 G in addition to the general lipid reactants; it was cardiolipin.

After it had been revealed that in *Ps. pyocyanea* phosphatides belonging to the group of ester phosphatides were predominating, we attempted to isolate lecithin and cephalin. The results are presented in Table II.

Table II

Lecithin and cephalin content of Ps. pyocyanea cultures of various ages

Age of culture, hours	Percentage values for total phosphatide*	
	Lecithin Cardiolipin	Cephalin Cardiolipin
8	89.8	10.2
18	87.2	12.8
48	87.1	12.9

* Data indicate average values obtained in two parallel cultivations

From Table II it is seen that in the phosphatides of the examined organism lecithin-type components comprised nearly 90 per cent. The cephalin content was somewhat over 10 per cent.

Results of gas chromatography. Fig. 2 and Table III reveal that palmitic acid was the main fatty acid component in all cultures. Fatty acids containing more than 16 carbon atoms were predominating. Those containing less than 16 carbon atoms were encountered in 10 per cent.

Cultures of various ages differed in the occurrence of a C_{19} cyclopropane ring-containing fatty acid (methyleneoctadecanoic acid). In 8 hour cultures this substance comprised 2 per cent of the total fatty acid content. In 18

Table III

Quantitative distribution of fatty acids in Ps. pyocyanea, per cent of total area

Serial number	Fatty acids	Designation	Age of culture, hours		
			8	18	48
1	n-Hexadecanoic acid — palmatic acid —	C_{16}	46.6	44.8	45.0
2	Octadecenoic acid	$C_{18:1}$	32.2	16.4	8 .1
3	Methylene-octadecanoic acid	$C_{19}\triangle$	2.0	20.9	25.4
4	n-Heptadecanoic acid	C_{17}	6.7	4.5	7.5
5	n-Dodecanoic acid — lauric acid —	C_{12}	5.0	4.0	4.7
6	Methylene-hexadecanoic acid	$C_{17}\triangle$	1.8	5.1	4.0
7	n-Pentadecanoic acid	C_{15}	2.5	2.0	1.8
8	n-Tetradecanoic acid — myristic acid —	C_{14}	1.2	0.7	1.1
9	n-Octadecanoic acid — stearic acid —	C_{18}	1.0	0.7	0.8
10	Undecenoic acid	$C_{11:1}$	0.4	0.5	0.7
11	Tridecenoic acid	$C_{13:1}$	0.5	+	0.4
12	Decenoic acid	$C_{10:1}$	+	+	+
	$C_{17}\triangle + C_{19}\triangle$		3.8	26.0	

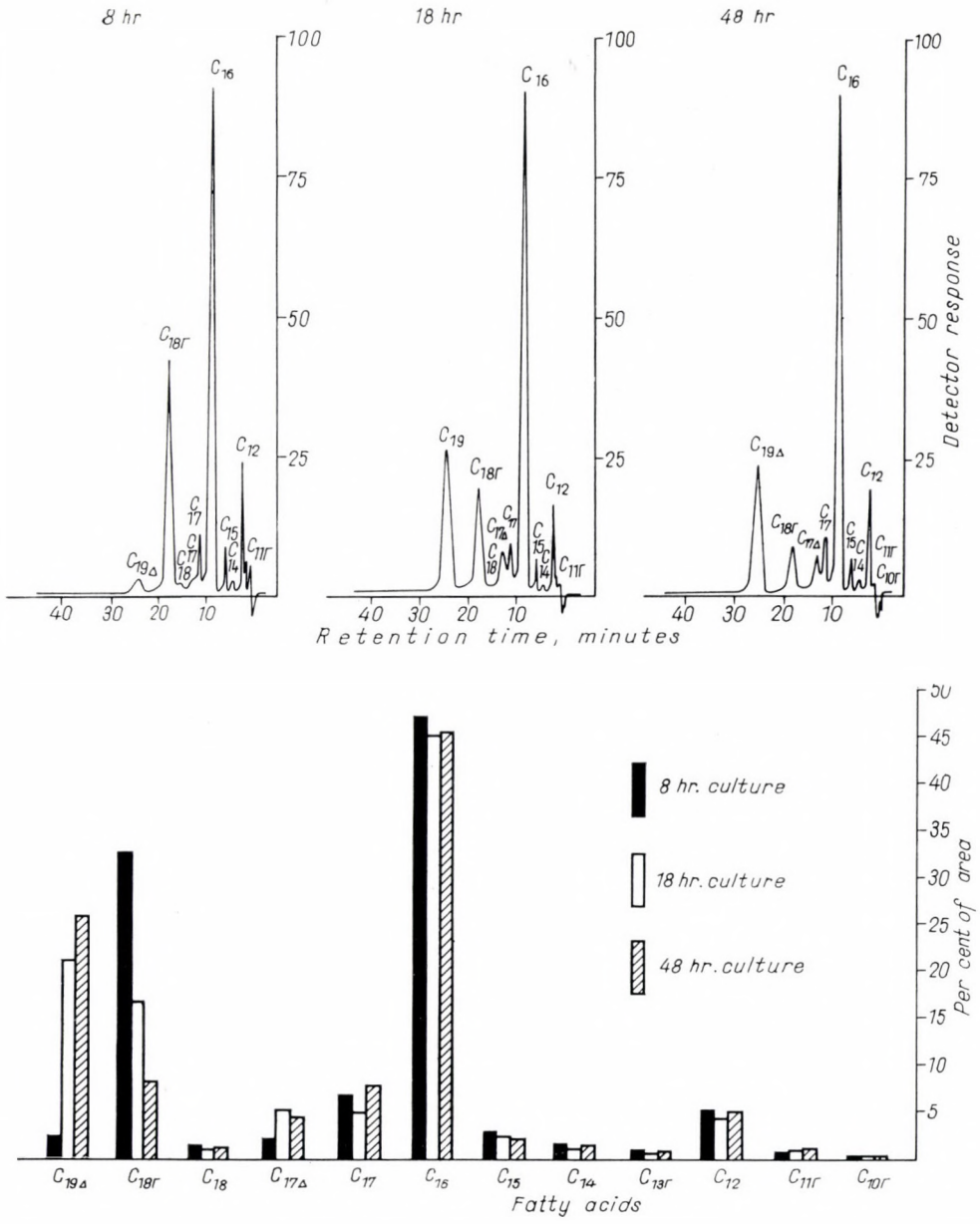

Fig. 2. Fatty acids in *Ps. pyocyanea*

and 48 hour cultures it amounted to 20.9 and 25.4 per cent, respectively. Thus, parallel with the cultures ageing, the amount of cyclopropane ring-containing fatty acids increased considerably.

Another finding was the variable occurrence in cultures of various ages of octadecanoic acid containing one unsaturated bond (32 per cent in 8 hour, 16.4 per cent in 18 hour, and 8.1 per cent in 48 hour cultures).

Discussion

In previous studies differences have been revealed in the phosphatide content and composition of three *Staph. aureus* strains with different antibiotic sensitivity [1,2]. With the broadenig of the resistance spectrum, an increase in cephalin-type, and a decrease in lecithin-type phosphatides has been observed. While an antibiotic sensitive *Staph. aureus* contained 88 per cent lecithin, in polyresistant cultures these substances were present in less than 70 per cent. These data have been confirmed by recent results showing a more complex composition of phosphatides in a *Ps. pyocyanea* strain with increased antibiotic resistance.

In the present experiments we have attempted to show whether the phosphatide content, number of phosphatide constituents and fatty acid composition differed in a given organism, if under standard experimental conditions the cultivation time only was altered.

The results have shown that the phosphatide content increases with the cultures ageing. The number of phosphatide components, however, remains unchanged. Prolongation of the incubation time does not induce the synthesis of new phosphatide components. Cultures of various ages differ but slightly as to the ratio of lecithin-type and cephalin-type components.

An interesting finding was revealed when *Ps. pyocyanea* was cultured in the presence of vitamin B_1. As compared to the 5.5 per cent value obtained with the control culture, the dried bacteria grown for 18 hours in the presence of vitamin B_1 contained 7 per cent phosphatide. There was a difference in the lecithin — cephalin ratio, too, as the lecithin content decreased to 72 per cent, and the cephalin content increased to 27 per cent, as compared to the 87 and 12 per cent values obtained with the strain grown in vitamin B_1-free medium.

As the examined *Ps. pyocyanea* strain was relatively sensitive to antibiotics, it stands to reason that the change in the phosphatide composition of the cell was due to some substrates. The entry of substrates into the cell is a function of reactivity with different phases. If more complex phases are encountered, the entry of certain molecules may decrease or cease.

Our recent results indicate that more complex phosphatides are present in *Ps. pyocyanea* cultures having a broader resistance spectrum [11].

Ps. pyocyanea cultures of different ages differed more definitely in fatty acid composition. The examined 3 cultures uniformly contained 12 fatty acids. Although during prolonged incubation the organism produced no further fatty acids, the ratio of these compounds varied with the age of the culture. Thus, the amount of a fatty acid containing C_{19} cyclopropane ring increased as incubation proceeded. The cause of this phenomenon is unknown. It is, however, known that older cultures are more resistant to antibiotics. In a previous paper [12] the same fatty acid has been shown to occur in considerable amounts in an antibiotic sensitive Proteus strain. Thus it may be assumed that this substance participates in the development of cell structure and resistance.

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ETUDE COMPARATIVE ENTRE LES SOUCHES BCG DE PARIS ET DE BUDAPEST

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Résumé. L'étude de l'allergie chez les sujets vaccinés de 1954 à 1957 a montré l'insuffisante efficacité du BCG alors préparé en Hongrie à partir d'une souche reçue en 1933 de l'Institut Pasteur de Paris. Il a d'abord été tenté d'améliorer les résultats en augmentant la concentration du vaccin. Mais l'expérimentation chez la souris et chez le cobaye a fait apparaître que la «sous-souche» de Budapest était d'une activité faible comparée à celle de la souche actuelle de l'Institut Pasteur. On a donc substitué cette dernière à la précédente pour la fabrication du vaccin hongrois à partir de 1960.

Il est reconnu aujourd'hui que des différences sensibles peuvent se manifester entre les souches BCG entretenues dans différents laboratoires et servant à la préparation du vaccin [1 à 9]. Ces différences peuvent aider à interpréter les variations observées dans les résultats de la vaccination par le BCG [10 à 13].

L'Institut d'Hygiène Publique de Budapest a reçu en 1933 la souche BCG 458/S2 de l'Institut Pasteur de Paris [14]. Entre 1955 et 1957 on a observé la diminution de l'effet allergisant de cette souche chez les sujets vaccinés. Cette constatation a été le point de départ d'un essai d'évaluation de la souche de Budapest dont nous avons cherché à comparer expérimentalement l'activité avec celle de la souche de l'Institut Pasteur de Paris.

Matériel et méthodes

(A) *Souches utilisées. BCG-Paris.* La souche BCG de l'Institut Pasteur de Paris communiquée au laboratoire du Centre International de l'Enfance en 1959 et entretenue sur milieu liquide de Dubos (Tween-albumine).

BCG-Budapest. Origine et entretien. La souche BCG n° 458/S2 est arrivée en Hongrie le 10 avril 1933, envoyée par l'Institut Pasteur de Paris. La souche a été entretenue de 1933 à 1935 à l'Institut de Microbiologie de Szeged, de 1935 à 1944 à l'Institut d'Hygiène Publique de Budapest, de 1944 à 1946 à l'Institut de Recherches de Biologie de Tihany et depuis 1946 de nouveau à l'Institut d'Hygiène Publique de Budapest. Les passages sur pomme de terre biliée ont été effectués toutes les 3 à 4 semaines et parallèlement les ensemencements sur pomme de terre glycérinée. Le passage sur Sauton a été réalisé à partir de la pomme de terre glycérinée 3 à 4 semaines après l'ensemencement. Pour la fabrication du vaccin, ont été employées des cultures sur Sauton âgées de 10 à 47 jours. D'après les protocoles, il a été observé quelquefois des accidents d'étuve et des anomalies de croissance de la souche.

De cette souche 458/S2 dérivent plusieurs sous-souches dénommées «sous-souches-Budapest», (BCG Bp.)

BCG Bp 332. La souche 458/S2 a été passée sur pomme de terre biliée 332 fois entre 1933 et 1959.

BCG Bp 226. Provient d'une culture lyophilisée en bouillon le 24 février 1948 après le 213e passage et remise en suspension le 2 janvier 1956 sous le nom de «souche 266».

BCG Bp 409. Provient d'une culture lyophilisée le 15 mai 1948 après le 217e passage et remise en suspension le 2 janvier 1956 sous le nom de «souche 409».

BCG Bp 410. Même origine que la souche 409 mais la remise en suspension est du 21 février 1958.

Pour réaliser les expériences comparatives, nous avons entretenu ces souches sur un milieu liquide de Dubos; repiquage tous les huit jours. Les vaccinations et les inoculations des animaux d'expérience ont été faites avec les cultures âgées de 8 à 10 jours.

Souche virulente. Pour l'injection d'épreuve, nous avons employé la souche H37Rv âgée de 8 à 10 jours, adaptée pour la souris, entretenue dans le laboratoire du CIE sur milieu de Dubos.

Détermination des unités vivantes. Pour les injections de BCG et pour les injections d'épreuve, le nombre des unités vivantes a été déterminé d'après la méthode de LEBLOIS [15].

(B) *Animaux d'expérience. Souris.* Swiss albinos de 25 à 30 jours. Inoculation de BCG par voie intraveineuse à la queue sous un volume de 0,2 ml. Dilutions faites en Sauton 1/4 avec 1 p. 1000 d'albumine bovine. Injection d'épreuve trois semaines après la vaccination en Sauton 1/4 avec 1 p. 1000 de sérum de lapin comme diluant.

Cobayes albinos de 400 à 500 g. Epilation, coupure des poils aux ciseaux. Avant l'expérience, on fait un test tuberculinique de 10 unités.

La répartition des animaux a été faite par tirage au sort. Les animaux ont été mis à un régime d'aliments «conditionnés» avec suppléments de carottes et de choux.

(C) *Comptes de colonies dans les organes.* Les souris ont été sacrifiées au chloroforme 3 semaines après injection d'épreuve de culture virulente diluée. Après une autopsie stérile, on broie la rate d'une part, les poumons d'autre part dans 3 ml de solution d'albumine bovine à 2 p. 1000 avec des broyeurs de Teflon. Les dilutions de broyats sont faites dans une solution d'albumine bovine à 0,1 p. 100. On ensemence 0,1 ml des dilutions appropriées sur les surfaces du milieu Jensen coagulé en tubes de Legroux. Les tubes sont placés à l'étuve à 37° et on effectue une lecture par semaine. Pour les comparaisons, a été retenu le nombre des colonies au 42e jour qui suit l'ensemencement.

(D) *Temps de survie.* Etudié comparativement dans des groupes de souris vaccinées, non vaccinées, après injection d'épreuve massive.

Résultats

Les tableaux I et II font apparaître à la fois, pour les souches étudiées, la multiplication du BCG seul dans les organes de la souris («test de virulence») et la protection contre l'injection d'épreuve («test de protection»).

Le tableau III donne les résultats d'une épreuve de «temps de survie».

Réaction locale chez le cobaye. Des groupes de 10 cobayes ont été injectés avec les souches BCG par voie intradermique sous un volume de 0,1 cc. Nous avons mesuré les diamètres des réactions locales jusqu'au 50e jour (tableau IV).

Discussion

En Hongrie, pour fabriquer le vaccin BCG, on a longtemps employé des cultures issues de la souche 458/S2 reçue de l'Institut Pasteur le 10 avril 1933.

Pour les nouveau-nés, la vaccination est obligatoire depuis 1954. La vaccination des enfants d'âge scolaire est pratiquée moins régulièrement. Les tests tuberculiniques post-vaccinaux ont montré que le vaccin préparé à partir de la souche BCG entretenue depuis 1933 à Budapest possède une capacité allergisante diminuée.

Tableau I

Multiplication du BCG dans les organes de la souris et effet d'une injection d'épreuve (H37Rv). Nombre des colonies obtenues à partir des organes

BCG UV	H37Rv UV	Organes	Dilution de broyat des organes	Nombre des colonies poussant aux dilutions indiquées sur cinq souris					$\bar{x}$
Paris 487.600	—	Poumon	10 ×	12	16	30	38	90	37,2
			100 ×	2	4	4	4	16	6,0
		Rate	10 ×	58	60	70	80	96	72,8
			100 ×	6	8	8	14	36	14,4
	11.508	Poumon	100 ×	0	0	0	5	10	3,0
			1000 ×	0	0	0	0	4	0,8
		Rate		non examinés					
Bp. 409 135.680	—	Poumon	10 ×	0	4	4	6	6	4,0
			100 ×	0	0	0	0	0	0,0
		Rate	10 ×	12	14	20	20	34	20,0
			100 ×	2	2	2	2	3	2,2
	11.508	Poumon	100 ×	14	20	24	36	98	38,4
			1000 ×	4	6	8	10	16	8,8
		Rate		non examinés					
—	11.508	Poumon	100 ×	21	50	80	92	100	68,6
			1000 ×	8	8	10	18	60	20,8
		Rate		non examinés					

— Vaccination par le BGG: 0,2 ml par voie i. v. d'une culture de Dubos âgée de 8 jours.
— Injection d'épreuve: H37Rv, 0,2 ml par voie i. v. d'une culture de Dubos âgée de 8 jours après 42 jours.
— 63 jours entre la vaccination et le sacrifice des animaux.
— 21 jours entre l'injection d'épreuve et le sacrifice des animaux.

D'après Flesch et Halász [16], l'allergie post-vaccinale ne dure que deux ans. Selon Stark [17] les nouveau-nés ont un pourcentage de 90 p. 100 mais l'allergie disparaît au bout de deux ans. Karossa-Pfeiffer et Bognár [18] ont observé que les enfants d'âge scolaire vaccinés en novembre et décembre de 1953 ont déjà perdu leur allergie postvaccinale dans une proportion de 20 p. 100 en 18 mois. Voith [19] a constaté un taux d'allergie de 80 p. 100 chez les écoliers un an après la vaccination. Szungyi [20] a trouvé une perte de l'allergie postvaccinale de 30 p. 100 au bout d'un an, 40 p. 100 au bout de deux ans et de 53 p. 100 au bout de trois ans. Lábady [21] a observé un taux d'allergie postvaccinale de 84 p. 100 chez les nouveau-nés. Szalatkay [22] donne le chiffre de 80 p. 100.

Tableau II

Multiplication du BCG dans les organes de la souris et effet d'une injection d'épreuve (H37Rv). Nombre des colonies obtenues à partir des organes

BCG UV	H37Rv UV	Organes	Dilution de broyat des organes	Nombre des colonies poussant aux dilutions indiquées sur cinq souris					$\bar{x}$
Paris 6.080	—	Poumon	10 ×	//	1	3	4	4	3,0
			100 ×	//	0	0	0	0	0,0
		Rate	10 ×	6	6	8	10	20	10,0
			100 ×	0	0	1	2	2	1,0
	20.160	Poumon	100 ×	//	10	12	16	24	15,5
			1000 ×	//	0	1	2	2	1,2
		Rate	100 ×	7	9	10	14	22	12,4
			1000 ×	1	2	2	2	3	2,0
Bp. 332 15.600	—	Poumon	10 ×	//	//	0	0	0	0,0
			100 ×	0	0	0	0	0	0,0
		Rate	10 ×	0	0	0	0	10	2,0
			100 ×	0	0	0	0	1	0,2
	20.160	Poumon	100 ×	//	//	40	100	100	80,0
			1000 ×	//	14	22	24	40	25,0
		Rate	100 ×	28	100	100	100	100	85,6
			1000 ×	16	22	24	32	46	28,0
—	20.160	Poumon	100 ×	//	//	58	100	100	86,0
			1000 ×	//	//	8	70	100	59,3
		Rate	100 ×	55	66	68	100	100	76,8
			1000 ×	8	10	12	14	16	12,0

— Vaccination par le BCG: 0,2 ml par voie i. v. d'une culture de Dubos âgée de 8 jours.
— Injection d'épreuve: H37Rv, 0,2 ml par voie i. v. d'une culture de Dubos âgée de 8 jours après 42 jours.
— 63 jours entre la vaccination et le sacrifice des animaux.
— 21 jours entre l'injection d'épreuve et le sacrifice des animaux.
/ / non évaluable pour raison technique

Comme résultat de ces observations, pour améliorer l'activité du vaccin du laboratoire du BCG de Budapest, il a été décidé d'augmenter la concentration du vaccin. Jusqu'au 20 décembre 1957 le vaccin était fabriqué à la concentration de 1,0 mg/ml, à partir de cette date la concentration a été doublée (2,0 mg/ml) [23]. Il est connu que la concentration optimale d'un vaccin est de 0,5 mg/ml à partir d'une souche ayant intégralement gardé sa virulence résiduelle originelle.

La valeur protective diminuée de l'ancienne souche BCG de Budapest rend vraisemblablement compte du fait que la morbidité par tuberculose, stagnant

Tableau III

Effet de la vaccination par le BCG. Temps de survie des souris après injection d'épreuve

BCG UV	H37Rv UV	Temps de survie												$\bar{x}$ harmonique	$\bar{x}$ et S. D. recipr.
Paris 4.480	6.788.000	30	31	37	39	40	44	47	48	55	60	60	//	42,0	23,8 5,8
Bp. 268 10.600	6.788.000	25	27	28	30	30	36	36	38	38	39	41	49	32,9	30,4 6,0
Bp. 332 9.836	6.788.000	16	24	24	24	25	27	27	30	41	41	46	48	27,6	36,0 12,0
Bp. 409 8.362	6.788.000	16	32	33	34	38	40	42	45	45	46	46	47	35,1	28,5 11,8
—	6.788.000	24	25	25	25	27	29	33	35	39	40	42	44	30,3	33,0 7,4

— Vaccination par le BCG: 0,2 ml par voie i. v. d'une culture de Dubos âgée de 8 jours
— Injection d'épreuve: H37Rv, 0,2 ml par voie i. v. d'une culture de Dubos âgée de 8 jours après 42 jours.
// non évaluable pour raison technique
— différence significative seulement entre les groupes de Paris et de contrôle.

Tableau IV

Réaction locale chez les cobayes provoquée par les souches BCG. Les chiffres donnés correspondent à la surface (en mm²). (Moyenne sur 10 animaux)

Jours / BCG UV	3		10	15	20	25	30	35	40	45	50
Bp. 332 18.400	1,98	4,16	6,72	14,32	11,96	7,46	5,53	2,63	0,88	0,23	0,0
Bp. 268 9.779	0,66	2,16	3,91	4,75	3,64	1,85	0,78	0,43	0,11	0,02	0,0
Bp. 409 11.400	1,10	2,63	5,18	6,79	5,16	2,30	1,98	0,80	0,25	0,06	0,0
Paris 10.200	0,50	2,01	5,50	12,66	20,36	25,27	20,63	12,65	8,44	4,73	1,50
Entre les souches Bp.	ns	ns	ns	DS	DS	DS	ns	ns	ns	ns	
Entre les souches Bp. et Paris	ns	ns	ns	ns	DS	DS	DS	DS	DS	DS	

— ns = avec analyse de variance: différence non significative
— DS = avec analyse de variance: différence significative

autour de 23 p. 10 000, n'a pas diminué de façon significative chez les enfants vaccinés entre 1954 et 1958.

D'autre part il n'a pas été observé de réactions locales et régionales postvaccinales appréciables malgré l'augmentation de la concentration. En réalité, de 1957 à 1959, on a appliqué des doses de vaccin quatre fois plus fortes au cours de la vaccination unilatérale et huit fois plus fortes au cours de la vaccination bilatérale simultanée. Mais il y a beaucoup de raisons de penser que même à dose élevée un BCG atténué à l'excès risque d'avoir une activité immunisante insuffisante.

Eu égard à nos résultats et aux données expérimentales publiées par plusieurs auteurs et nous rangeant aux conclusions de la Conférence Internationale Technique du BCG de 1956, nous employons depuis 1960 à Budapest la souche actuelle de l'Institut Pasteur pour fabriquer le vaccin. A partir de cette souche, nous préparons un vaccin liquide de concentration 0,4 mg/ml. L'allergie postvaccinale contrôlée chez les nouveau-nés avec le timbre tuberculinique (Human «Forte») montre un taux d'allergie de 93—95 p. 100.

Depuis le changement de la souche, 1 000 500 vaccinations ont été réalisées en Hongrie. Avant 1959 la morbidité par tuberculose des enfants de 0 à 14 ans était de 23 p. 10 000. Au cours de ces quatre dernières années la morbidité des enfants a diminué de 87 p. 100 et à la fin de 1963 elle a été de 3,1 p. 10 000. LUGOSI [24].

D'autres expériences nous ont permis récemment de vérifier la diminution de la valeur immunobiologique de l'ancienne sous-souche BCG de Budapest. LUGOSI *et al.* [25].

Analyses statistiques : Mme E. ORSSAUD (CIE), MM I. JUVANCZ et P. CSÁKI, Service de Biométrie de l'Académie des Sciences Hongroises auxquels les auteurs adressent leurs remerciements.

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HYPOTHERMIA AND HORSE SERUM ANAPHYLAXIS

By

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Summary. Guinea pigs sensitized with horse serum were challenged with various serum fractions. The most definite anaphylactogenic effect was exerted by the β-globulin fraction. In the hypothermic state, sensitized animals could be desensitized against weak, but not against strong anaphylactogenic fractions.

In a previous report [1] it has been shown that in the guinea pig anaphylactic shock can be prevented by decreasing body temperature. It has also been shown that in some respects there is a difference between the behaviour of animals sensitized with ovalbumin and with horse serum [2]. In these experiments the challenging dose was administered to guinea pigs sensitized with ovalbumin which had been cooled to 24—26°C. The animals were then brought to normal temperature and a second eliciting dose was injected. Under these conditions no anaphylactic shock was seen to develop. In contrast, when similar experiments were carried out in animals sensitized with horse serum, the second eliciting dose, given in the normothermic state, induced severe or even lethal reactions. In other words, desensitization was successful only against ovalbumin, but not against horse serum.

This paper gives an account of experiments in which we examined the role in the said phenomenon of various horse serum fractions.

Materials and methods

Male guinea pigs weighing 300 to 350 g were sensitized with two 0.2 ml subcutaneous doses of native horse serum. Various fractions were prepared from horse serum by Sober and Peterson's method elaborated for human serum [3]. This procedure yielded 4 fractions: α, β and γ-globulin and albumin. Fractionation was performed by the use of dimethyl-amido-diethyl cellulose anion exchange resin at 0.0175—0.4 *M* phosphate concentrations, pH 6.3—5.2 and 5°C. The obtained fractions were purified by precipitation with ammonium sulphate. Homogeneity of the fractions was checked with an Antweiler microelectrophoretic apparatus (pH 8.6; barbiturate buffer). As shown in Fig. 1, the β and γ-globulin fractions were homogeneous; the albumin fraction contained pre-albumin and in the α-globulin fraction both α_1 and α_2 groups were present.

The guinea pigs were anaesthetized with 90 mg/kg subcutaneous doses of phenobarbital and then they were cooled by means of salted ice placed in plastic sacs. According to Keszty*ű*s *et al.* [4], phenobarbital exerts no influence on the anaphylactic shock of guinea pigs. Rewarming of the animals was begun at the cardial region; normal body temperature was reached approximately after 3 hours. Rectal temperature was recorded with a thermistor thermometer.

According to the severity of anaphylaxis, four degrees were distinguished; mild (sneezing, ruffling of hair, rubbing), moderate (passing of urine and faeces, dyspnoea), severe (falling on the side, severe dyspnoea), and lethal.

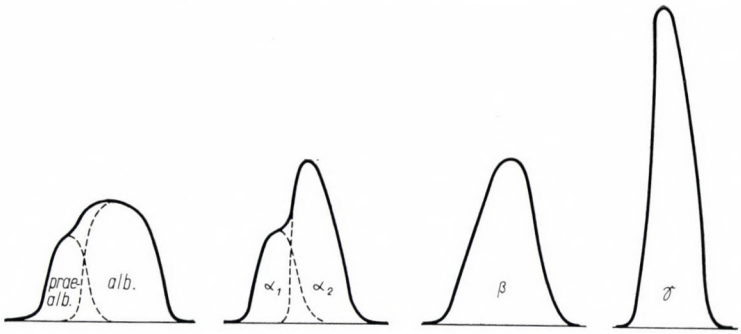

Fig. 1. Electrophoresis of horse serum fractions. Antweiler apparatus, pH 8.6; barbiturate buffer

Results and discussion

In the first series of experiments the anaphylactogenic effect of the four serum fractions was examined. The guinea pigs were sensitized with horse

Table I

Anaphylactogenic effect of horse serum fractions

Eliciting injection	Protein content of eliciting injection, mg	Result
Horse serum	48	Death in 5 min. (10)*
Horse serum α-globulin fraction	5	Moderate shock (5)
	7.5	Death in 5 min. (2) Moderate shock (8)
Horse serum β-globulin fraction	5	Death in 5 min. (1) Moderate shock (4)
	7.5	Death in 5 min. (3) Severe shock (7)
Horse serum γ-globulin fraction	5	Mild shock (5)
	7.5	Mild shock (10)
Horse serum albumin fraction	10	Moderate shock (10)
	15	Moderate shock (5)

* Figures in bracket indicate the number of animals. Guinea pigs were sensitized with horse serum; challenging dose was given 3 weeks later.

serum, then after 3 weeks the various fractions were administered via the jugular vein. As shown in Table I, whole serum caused within 5 minutes lethal anaphylactic shock in all animals. This indicates that the guinea pigs had been sensitized against horse serum. Of the fractions γ-globulin and albumin were less definitely anaphylactogenic; β-globulin caused the most severe reactions.

When subsequent intrajugular injections of whole horse serum were given to the surviving guinea pigs, lethal anaphylactic shock was observed mainly among animals which had been challenged previously with γ-globulin or albumin. This finding also indicated that these fractions exerted the smallest anaphylactogenic and desensitizing activity. Our results agree with those of ANDZHAPARIDZE [5], who reported that β-globulin was the fraction causing the most definite anaphylaxis.

In subsequent experiments the effect of serum fractions on guinea pigs, in which anaphylaxis had been prevented by hypothermia, was examined. The animals were sensitized with horse serum. After 3 weeks they were cooled to 25—26°C and challenged with horse serum. Then they were rewarmed to normal temperature and approximately after 12 hours injected with various serum fractions and with whole serum as the control.

Table II

Anaphylactogenic effect of horse serum fractions after desensitization in hypothermia

Second eliciting injection	Protein content of 2nd eliciting injection, mg	Result
Horse serum	48	Death in 5 min. (6) Severe shock (4)
Horse serum α- globulin fraction	7.5	Severe shock (2) Mild shock (6) Symptompless (7)
Horse serum β-globulin fraction	7.5	Death in 5 min. (3) Moderate shock (9) Symptomless (3)
Horse serum γ-globulin fraction	7.5	Symptomless (8)
Horse serum albumin fraction	10	Symptomless (8)

Figures in bracket indicate the number of animals. Guinea pigs were senzitized with horse serum. The first eliciting dose (horse serum containing 48 mg protein) was given in hypothermia (25—26°C). The second eliciting dose was administered 12 hrs. later in normothermia.

As shown in Table II, the second dose of horse serum caused lethal or severe anaphylaxis. Of the fractions, γ-globulin and albumin produced no reaction; α-and mainly β-globulin caused severe symptoms.

The present experiments have shown that horse serum components differ in anaphylactogenicity. It has also been revealed that in the hypothermic state desensitization is successful only against weak anaphylactogenic fractions. In the case of highly anaphylactogenic fractions a partial antigen—antibody binding apparently occurs in the cooled animals. Our results indicate that the most definitely anaphylactogenic fraction is β-globulin, and that desensitization of hypothermic guinea pigs is less successful against this component.

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ATYPICAL MYCOBACTERIA IN MONKEYS

By

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Summary. From two tuberculin-positive monkeys *(Macacus rhesus* and *Papio hamadrias)* shortly after the animals had been imported, two Mycobacterium strains were isolated: M2 and Zol. Strain M2 is a *M. tuberculosis* strain of attenuated virulence. Strain Zol is non-pigmented; in biochemical, immunogenic and antigenic properties it differs sharply from *M. tuberculosis.* The strain did not correspond to mycobacteria belonging to the Battey strains or to saprophytic mycobacteria.

Tuberculosis frequently occurs among monkeys, and it is generally accepted that monkeys in captivity acquire infection from man [3, 7].

Numerous institutes are keeping considerable numbers of monkeys for virological purposes, *e.g.* poliomyelitis vaccine production, examination of spontaneous viruses of these animals, etc. As the laboratories usually receive the monkeys shortly after they have been captured, it is unlikely that such animals might have been infected by man. Nevertheless, many of them have tuberculosis and discarding the infected animals on the basis of the tuberculin test is a generally adopted method. But whether the tuberculin positive monkeys are really infected with *M. tuberculosis*, is a point on which there are no data in the literature.

Considering that most of the experimental monkeys come from India, it is of interest that Mitchison [6], Bhatia [1] isolated *M. tuberculosis* strains of attenuated virulence from the sputum of Indian patients who had received no chemotherapeutic drugs. The occurrence of atypical mycobacteria in man called attention to the pathogenic role of such organisms and their relationship to *M. tuberculosis* [4, 8, 10].

Material and methods

Two Mycobacterium strains isolated from tuberculin positive monkeys were examined. Strain M2 was isolated from a swollen inguinal lymph gland of a *Macacus rhesus* monkey, imported from India. Strain Zol was isolated by Dr. Pekerman by puncturing a slightly swollen axillary lymph gland of a baboon *(Papio hamadrias)*. The latter animal was imported from Abyssinia to the Suhum (USSR) experimental monkey station.

The tuberculin test was performed with 100 I. U. by the intradermal method over the eyebrows. Readings were made after 72 hours. The two strains were examined for cultural, biochemical, pathogenic and immunogenic properties. In addition, the antigenic structure of strain Zol was also studied (Gimpl). Examination of cultural properties included the growth

on different media and pigment production. Of the biochemical reactions, niacin production, catalase, peroxidase, nitrate reductase, aryl sulphatase activities and the acylamidase spectrum [2] were examined. In addition, the respiration-promoting effect of sodium benzoate and salicylate was studied. Pathogenicity was tested by giving doses of 1 mg intravenously to 5 white mice, 1 mg subcutaneously to 3 guinea pigs and 5—10—20 mg intravenously to rabbits (each dose to 3 animals). Immunogenicity was investigated by inoculating guinea pigs with 1 mg subcutaneously; for comparison another group of animals received BCG. After 4—6 weeks all these animals and a control group were challenged with 0.00001 mg of strain H37Rv subcutaneously. Antigenic structure was examined by agar gel precipitation [5]. For comparison *M. tuberculosis* and *M. avium* strains were used.

Results

Strain M2 shows the characteristic cultural properties of *M. tuberculosis*. It produces at 37°C rough colonies and is an R variant. Biochemical properties (Table I): It gives an intensive niacin reaction; produces catalase, peroxidase, nitrate reductase and, of the acylamidases, urease and nicotinamidase. Aryl-

Table I

Biochemical properties of the monkey strains

	Strains		
	M. tuberc. BCG	M2	Zol
Niacin	+	++	—
Catalase	+	+	+
Peroxydase	+	+	+
Nitrate reductase	—	+	+
Arylsulphatase	+	—	+
Urease	+	+	+
Nicotinamidase	+	+	+
Pyrazinamidase	—	—	+
Salicylate effect	+	—	—
Benzoate effect	—	—	—

sulphatase is not produced. Its sensitivity to chemotherapeutic substances corresponding to that of the tubercle bacillus (Table II), being sensitive to 1 μg/ml of streptomycin, isoniazid and PAS. Thiosemicarbazone inhibited growth only at 50 μg/ml concentration. Pathogenicity to animals: 5 mg culture gave rise in the rabbit's lung to numerous grey, non-necrotic foci after 79 days; in the guinea pig, at the site of the inoculation, an abscess was produced without any lesions in other organs. For mice some degree of pathogenicity was observed, as after 73 days in the lungs numerous foci developed and in some cases a hypertrophy of the spleen occurred. Strain M2 possesses a high

Fig. 1

degree of immunizing capacity against tuberculosis (Table III). Superinfection of immunized animals gave rise only to lesions in the regional lymph glands, whereas in the control animals grave tuberculosis developed. This strain is therefore a typical tubercle bacillus of low virulence in experimental animals.

Strain Zol produces no pigment, and does not grow at room temperature. At 37°C on glycerol medium it forms smooth S colonies after 7—19 days. Niacin is not produced. The strain possesses catalase, peroxidase, nitrate reductase and arylsulphatase. Of the acylamidases urease, nicotinamidase and pyrazin-amidase are formed. The strain is resistant to streptomycin, isoniazid, PAS and thiosemicarbazone, and shows growth on media containing

Table II

Growth of strains isolated from monkeys on media with chomotherapeutic substances at different concentrations

Strains	Streptomycin			IHN			PAS			Thiosemicarbazone		
	1	10	50	1	10	50	1	10	50	1	10	50
	μg/ml			μg/ml			μg/ml			μg/ml		
M 2	—	—	—	—	—	—	—	—	—	—	—	—
Zol	+	+	+	+	+	+	+	+	+	+	+	±

± = partial resistance

Table III

Immunogenic properties of the monkey strains

Strain	No. of animals tested	Tbc index*
M 2	8	4.5
BCG	9	7.8
— (control)	10	19.3
Zol	9	15.8
BCG	10	2.5
— (control)	9	12.3

+ maximum involvement = 22

these substances at 50 μg/ml concentration. It causes no pathological changes in rabbits, mice and guinea pigs. Strain Zol exhibits no immunizing capacity against tuberculosis (Table III). Guinea pigs inoculated with this strain similarly to the non-immunized control animals, became severely ill after superinfection with virulent tubercle bacillus, with a tbc index of 15.8 the animals vaccinated by BCG have had an index of 2.5. The antigenic structure of strain Zol is considerably different from that of human and avian *M. tuberculosis* strains, H 37 Rv and Kirchberg which possess only 2 common antigens out of 10 (Fig. 1).

Discussion

It has been found that the two monkeys from which our strains had been isolated, were slightly sensitive to tuberculin and had not been infected by typical tubercle bacilli. At autopsy the organs of the Macacus rhesus monkey showed no tuberculous alterations. It was striking that in both cases the strains were isolated from the subcutaneous axillary or inguinal lymph glands. This finding suggests that the bacteria had gained access through the skin and not by the respiratory or alimentary route.

Strain M2 was a typical attenuated tubercle bacillus. This was indicated by its cultural properties, niacin reaction and enzyme activity, streptomycin, PAS and isoniazid sensitivity and its immunizing capacity against tuberculosis. This finding is interesting since in India, where our monkey originated from, similar strains had been isolated from man by MITCHISON. The question might arise whether a laboratory infection with our attenuated strain 115 [9], or BCG might have occurred. This possibility, however, is excluded by the fact that the animal was tuberculin positive when it was received and, also, that the strain was isolated as soon as 2 months thereafter. In addition, the strain differed from strains 115 and BCG in its negative arylsulphatase and salicylate

reactions. Its mouse pathogenicity was higher than that of BCG, but lower than that of strain 115.

Strain Zol basically differs from *M. tuberculosis* and, according to RUNYON's classification of atypical strains, it would belong to the group of non-chromogenic or Battey strains. However, it cannot be fitted into this group, as the Battey strains can hardly be differentiated from avian strains and are capable of immunizing against tuberculosis. The antigenic and immunogenic properties of strain Zol differ definitely both from avian and mammalian *M. tuberculosis*. Nevertheless, as it does not grow below 37°C, the strain cannot be regarded as a saprophytic organism.

Our strains caused no severe pathological changes in monkeys but, probably after penetrating through the skin, gave rise to a latent infection. From our findings it can be concluded that large-scale investigations are desirable into this point, as at present we have no data on the frequency of mycobacterial infections among wild monkeys.

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BIOCHEMICAL CHLORINATION INHERITANCE IN STREPTOMYCES AUREOFACIENS

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Summary. Chlorination inheritance in *Streptomyces aureofaciens* has been studied. Mixed growths started from conidial suspensions of chlorinating (cle^+) and non-chlorinating (cle^-) biochemical mutants always yielded recombinants with high chlorinating activity (92—100 per cent of the total tetracycline potency). The new recombinants fell into two groups, viz. chloride scavenger cultures with 100 per cent chlorinating activity and chloride non-scavenger cultures with 92—98 per cent chlorinating activity.

Acridine orange tests indicated the chromosomal origin of the chlorination gene.

The tested auxotroph combinations could be divided into three groups. The first group yielded high (2.03×10^{-4}), the second low (0.015×10^{-4}) recombination frequencies. The third group comprised intersterile cultures. The finding can be explained by assuming a sexual polarity in Streptomycetes.

Certain *S. aureofaciens* strains have been described which are uncapable of biological chlorination, and which chlorine-containing medium produce only tetracycline [1, 2]. In the present experiments mutants synthesizing chlorinated tetracyclines and mutants failing to exert any chlorinating activity were used. The inheritance of chlorination was studied by gene recombination and acridine organe treatment. In addition, some data have been obtained as to the mechanism of gene recombination.

In studies on gene recombination in *S. aureofaciens* [3—5] it has been shown that prototrophic recombinants originating from certain complementary auxotrophs produce considerably higher amounts of total tetracycline than any of the parent strains [4]. In the present experiments the conditions responsible for chlorination inheritance in recombinants have been studied, but no detailed investigations have been performed into the association between tetracycline biosynthesis and gene recombination. It should, however, be noted that tetracycline production, as compared to the antibiotic-producing capacity of auxotrophs, was restitued in their recombinant progenies.

In order to determine whether the genetic unit responsible for chlorination is chromosomal or episomic in origin, experiments with acridine orange were performed. It is known that episomic units, in other words factors of cytoplasmic nature, such as factor F in *Escherichia coli* [6], the drug-resistance factors in Enterobacteriaceae [7, 8] and the respiratory capability factor in yeasts [9] can be altered or eliminated by acridine treatment.

Materials and methods

Chlortetracycline-producing B-28 [10] and tetracycline-producing CDSD-314 [1] *S. aureofaciens* strains were used as parent cultures. From these strains two groups of biochemical mutants were obtained on treatment with different mutagenic agents [4]. The mutants are presented in Table I.

Conidia for recombination experiments were obtained from cultures grown at 27°C for 14—20 days on Czapek-Dox medium supplemented with the factor(s) required by the corresponding auxotrophs. Conidial suspensions of the complementary auxotrophs were mixed (1 : 1) then plated on supplemented Czapek-Dox medium.

After incubation the conidia from isolated heterokaryotic colonies were transferred to minimal medium. The prototrophic culture was examined for chlorinating activity after incubation at 27°C for 14—20 days.

In experiments with acridine orange corn steep medium was also used. The medium contained corn steep liquor (50 per cent dry weight), 0.65 per cent; sucrose, 1.0 per cent; KH_2PO_4, 0.4 per cent; $(NH_4)_2HPO_4$, 0.25 per cent; $MgSO_4$, 0.25 per cent; agar, 2.5 per cent; pH 6.8.

Chlorination by original cultures and recombinants was examined in shaken cultures incubated at 27°C. The inoculum medium contained starch, 2.0 per cent; corn steep liquor (50 per cent dry weight), 4.0 per cent; $CaCO_3$, 0.5 per cent; pH 6.8. Ingredients of the fermentation medium were: starch, 3 per cent; corn steep liquor (50 per cent dry weight), 1.2 per cent; NH_4NO_3, 0.7 per cent; NaCl, 0.6 per cent; $CaCO_3$, 0.6 per cent; $CuSO_4$, 0.004 per cent; pH 6.2.

The produced antibiotics were separated into chlorinated and non-chlorinated tetracyclines by paper chromatography using the double solvent method. As solvents ethylacetate saturated with pH 4.7 McIlvain buffer and chloroform: butanol (9 : 1) saturated with aqueous 0.3 *M* H_3PO_4 and 0.1 per cent trichloracetic acid solution were used [11, 12]. Paper sheets were impregnated with 1.0 per cent Selecton B.

Table I

Streptomyces aureofaciens strain

Strain	Genotype	Frequency of spontaneous back mutation, $\times 10^{-6}$	Strain	Genotype	Frequency of spontaneous back mutation $\times 10^{-6}$
B—28	arg$^+$ met$^+$ cys$^+$ asp$^+$ am$^+$ cle$^+$	—	CDSD-314	arg$^+$ met$^+$ thi$^+$ pi$^+$ am$^+$ cle$^-$	—
N—2	arg$^-$ cle$^+$	0.035	N—23	arg$^-$ cle$^-$	0.14
N—4	met$^-$ cle$^+$	0.28	N—25	met$^-$ cle$^-$	0.35
N—6	arg$^-$ cle$^+$ cys$^-$	0.00011	N—29	arg$^-$ cle$^-$	0.092
N—9	arg$^-$ cle$^-$	0.041	N—30	arg$^-$ cle$^-$ am$^-$	0.0055
N—10	cys$^-$ cle$^+$	0.81	N—32	arg$^-$ cle$^-$ am$^-$	0.0082
N—11	met$^-$ cle$^+$ am$^-$	0.0083	N—33	thi$^-$ cle$^-$ pi$^-$	0.0033
N—16	met$^-$ cle$^+$ asp$^-$	0.025			
N—18	cys$^-$ cle$^+$ asp$^-$	0.0052			

Abbreaviations: arg$^-$ arginine, met$^-$ methionine, am$^-$ amino nitrogen, asp$^-$ asparagine, thi$^-$ thiamine, pi$^-$ pyridoxine requiring mutants; cle$^-$ no chlorinating activity (tetracycline production), cle$^+$ chlorinating activity (chlortetracycline production).

Acridine orange treatment was carried out as follows. To the supplemented liquid minimal medium sterile acridine orange solution was added so as to give a final concentration of 2 to 10 μg/ml. Then the medium was seeded with conidial suspension and incubated under shaking at 27°C for 5 days. The conidia were separated by filtration and the mycelium was washed twice by centrifugation. The mycelium was then resuspended in pH 6.8 isotonic buffer and disintegrated in a cold blendor. Dilutions of the mycelial fragments were streaked onto corn steep agar. The developing colonies were transferred again to corn steep agar and the subcultures were examined as described above.

Table II

Heterokaryotic colonies obtained from auxotrophic combinations

Crosses	Genotype	No. of colonies*	Crosses	Genotype	No. of colonies*
N—2 N—25	arg⁻ cle⁺ met⁻ cle⁻	0	N—11 N—23	met⁻ am⁻ cle⁺ arg⁻ cle⁻	109
N—2 N—33	arg⁻ cle⁺ thi⁻ pi⁻ cle⁻	285	N—11 N—29	met⁻ am⁻ cle⁺ arg⁻ cle⁻	94
N—4 N—23	met⁻ cle⁺ arg⁻ cle⁻	390	N—11 N—33	met⁻ am⁻ cle⁺ thi⁻ pi⁻ cle⁻	105
N—4 N—29	met⁻ cle⁺ arg⁻ cle⁻	194	N—16 N—23	met⁻ asp⁻ cle⁺ arg⁻ cle⁻	89
N—4 N—30	met⁻ cle⁺ arg⁻ am⁻ cle⁻	2.5	N—16 N—29	met⁻ asp⁻ cle⁺ arg⁻ cle⁻	138
N—4 N—32	met⁻ cle⁺ arg⁻ am⁻ cle⁻	1.3	N—16 N—30	met⁻ asp⁻ cle⁺ arg⁻ am⁻ cle⁻	0
N—4 N—33	met⁻ cle⁺ thi⁻ pi⁻ cle⁻	312	N—16 N—32	met⁻ asp⁻ cle⁺ arg⁻ am⁻ cle⁻	2.2
N—6 N—25	arg⁻ cys⁻ cle⁺ met⁻ cle⁻	1.3	N—16 N—33	met⁻ sap⁻ cle⁺ thi⁻ pi⁻ cle⁻	136
N—6 N—33	arg⁻ cys⁻ cle⁺ thi⁻ pi⁻ cle⁻	248	N—18 N—23	cys⁻ asp⁻ cle⁺ arg⁻ cle⁻	75
N—9 N—25	arg⁻ cle⁺ met⁻ cle⁻	0	N—18 N—25	cys⁻ asp⁻ cle⁺ met⁻ cle⁻	0
N—9 N—33	arg⁻ cle⁺ thi⁻ pi⁻ cle⁻	178	N—18 N—29	cys⁻ asp⁻ cle⁺ arg⁻ cle⁻	117
N—10 N—23	cys⁻ cle⁺ arg⁻ cle⁺	415	N—18 N—30	cys⁻ asp⁻ cle⁺ arg⁻ am⁻ cle⁻	1.5
N—10 N—25	cys⁻ cle⁺ met⁻ cle⁻	0.4	N—18 N—32	cys⁻ asp⁻ cle⁺ arg⁻ am⁻ cle⁻	1.4
N—10 N—29	cys⁻ cle⁺ arg⁻ cle⁺	104	N—18 N—33	cys⁻ asp⁻ cle⁺ thi⁻ pi⁻ cle⁻	147
N—10 N—30	cys⁻ cle⁺ arg⁻ am⁻ cle⁻	2.2			
N—10 N—32	cys⁻ cle⁺ arg⁻ am⁻ cle⁻	0.7			
N—10 N—33	cys⁻ cle⁺ thi⁻ pi⁻ cle⁻	505			

* Average result of 2 to 5 experiments. Number of colonies is expressed in relative to 10^6 conidia. For the abbreviations, see Table I.

Results

Conidia of chlortetracycline-producing (cle^+) and tetracycline-producing (cle^-) biochemical auxotrophs were grown in mixed culture on minimal medium supplemented with low concentrations (10^{-5} to 10^{-8} *M*) of the corresponding growth factors. After 14—18 days conidia of heterokaryotic colonies were transferred to minimal medium [4]. Table II presents the number of recombinant colonies per 10^6 conidia obtained as an average result of 2 to 5 experiments.

It is seen that in some combinations high numbers of heterokaryotic recombinant colonies had developed. Sometimes, however, no successful crossing was observed.

Conidia from heterokaryotic colonies were transferred to minimal medium. The chlorinating activity of the prototrophic recombinants was examined after 14 to 18 days of incubation. The results are shown in Table III.

Recombinants failing to exert chlorination were not recovered. It should be noted that the majority of recombinants produced definite amounts of nonchlorinated antibiotic (tetracycline). Only chlorinated antibiotic was produced by recombinants originating from the two "donors" N-11 and N-18. It should be noted that each of the 8 auxotrophic "donors" used showed a 100 per cent chlorinating activity.

Table III

Chlorination by recombinants

Chlorinating (cle^+) parent	No. of recombinants	Chlorinating activity, per cent		
		100	92—98	<92
N—2	30	—	30	—
N—4	45	—	45	—
N—6	25	—	25	—
N—9	20	—	20	—
N—10	30	—	30	—
N—11	35	35	—	—
N—16	40	—	40	—
N—18	55	20	35	—

In subsequent experiments subcultures of 4 auxotrophic combinations were examined. Three heterokaryotic colonies of each combination were suspended and streaked on minimal medium. Isolated prototrophic recombinant colonies were further subcultured on minimal medium and examined for chlorinating activity. The results are shown in Table IV. It is seen that no

Table IV

Chlorination by subcultures of recombinants

Parental auxotrophic strains of recombinants	No. of subcultures	Chlorinating activity, per cent		
		100	92–98	<92
N—6 × N—33	190	—	190	—
N—11 × N—33	225	225	—	—
N—18 × N—29	150	150	—	—
N—18 × N—33	120	—	120	—

cultures with considerably decreased or ceased chlorinating activity were encountered.

In further experiments the effect of acridine orange on chlorination by wild-type parent strains and by biochemical mutants was examined. Mycelial fragments after acridine orange treatment were streaked on complete and minimal media. The mutagenic effect was estimated from the number of deficiency back mutations. The results (Table V) indicated that acridine orange had no influence on chlorination either in wild-type or in deficient mutants. A slight mutagenic action of the dye was observed.

Discussions

It has been shown that on crossing in suitable combinations, *S. aureofaciens* auxotrophs produce prototrophic recombinants. Data reported previously indicated that in biochemical mutants the frequency of gene recombinations was low (8.5×10^{-5}) [4]. In the present studies some auxotrophic recombinations showed considerably higher frequencies. As seen in Table II, on the basis of recombination frequency the cultures tested could be divided into two groups (0.015×10^{-4} and 2.03×10^{-4}). A third group comprised intersterile strains, which, under the experimental conditions, were unable to produce heterokaryotic colonies from mixed conidial suspensions. Our results are suggestive of the phenomenon observed in *S. violacoeruber* by BRADLEY and ANDERSON [13] and confirm the assumption of these authors that streptomycetes contain a specific "compatibility system" which controls recombination. The "compatibility system" (recombination factor) may indicate a sexual polarity in these organisms. The differences in recombination frequency revealed in these experiments may be explained by a sexual difference between the used *S. aureofaciens* cultures. Our studies on sexual polarity (unpublished data) indicated a sexual differentiation in *S. aureofaciens*. A similar finding was revealed by SERMONTI and CASCIANO in *S. coelicolor* (or *S. violaceoruber*) [15].

Chlorination was examined in prototrophic recombinants only as the used selective medium did not allow the isolation of other recombinant groups. It is, however, evident, that omission of nutritionally deficient recombinants (which exerted a considerably decreased total tetracycline-producing capacity) caused no error in the evaluation of results concerning chlorination inheritance. The mechanism of chlorination inheritance is presumably identical in auxotrophic and in other, not examined, recombinant groups.

A high chlorinating activity was evident in all recombinants. No recombinants with significantly decreased or ceased activity were encountered.

Similar results were obtained when the chlorinating activity of prototrophic cultures derived from heterokaryotic colonies was examined. ALIKHANIAN and BORISOVA [5] showed that segregation of *S. aureofaciens* from heterokaryotic colonies occurred in 0.02—1.5 per cent. In our experiments neither of the relatively large number of subcultures examined differed in chlorinating activity from the directly obtained recombinant cultures. As shown in Table IV, the activity of the former cultures was between 92 and 100 per cent. Accordingly, a subsequent loss by segregation of the regained activity has not been observed.

It is notable that highly active recombinants fell into two groups. Members of group 1 exerted a 100 per cent chlorinating activity, that is, they produced only chlortetracycline. Therefore these cultures corresponded to "chloride scavenger" strains [2, 16]. Cultures belonging to group 2, which produced 2 to 8 per cent non-chlorinated tetracycline by the end of fermentation, were regarded as "chloride non-scavenger" strains.

Among group 1 strains the rate of tetracycline biosynthesis does not exceed the rate of chlorination, that is, chlorine is incorporated in every tetracycline molecule. In contrast, in group 2 strains, because of a biochemical or genetic "disorder", the rate of chlorination is lower and, therefore, non-chlorinated tetracyclines also appear by the end of fermentation. Mutants unable to exert any chlorinating activity are deficient in the biosynthesis of a certain enzyme. This agent (probably a copper oxidase-type enzyme) was, however, invariably present in our recombinants, which all regained an essentially complete chlorinating activity.

In view of the genetically regained chlorination, from the obtained quantitative difference it may be assumed that recombination of genes responsible for tetracycline biosynthesis may vary with different auxotroph combinations.

The slight mutagenic effect of acridine orange reported in a previous paper [14] has also been demonstrated in the present experiments (Table V). The dye was ineffective on chlorination inheritance. It has been shown that in Streptomycetes acridine derivatives induce the loss of a gene which presumably is cytoplasmic in nature, and is responsible for tyrosinase activity.

As compared to the control, in the treated culture the frequency of tyrosinase deficient mutations was about 20 fold [17]. In our experiments chlorination inheritance was unaffected by acridine orange. Therefore, this structural gene was found to be a chromosomal factor.

Table V

Effect of acridine orange on chlorination

Strain	No. of cultures				Back mutation in treated cultures $\times 10^{-6}$	Spontaneous back mutat on in untreated cultures $\times 10^{-6}$
	Treated		Untreated			
	cle+	cle−	cle+	cle−		
B—28	180	—	20	—	—	—
CDSD—314	—	40	—	20	—	—
N—6	60	—	20	—	0.32	0.28
N—9	80	—	20	—	2.97	0.041
N—11	40	—	20	—	0.012	0.0083
N—29	—	20	—	20	0.22	0.092
N—33	—	20	—	20	0.085	0.0033

We have shown that the lack of chlorination is a rather stable genetic property of mutants. In our material not a single subculture occurred among thousands of tetracycline producing strains obtained by natural selection that would have regained chlorinating activity by back mutation.

These data and other experiments carried out with other mutagenic agents indicated that chlorination deficiency is similar to auxotrophy, and the gene for chlorination activity occurs in genetic interactions as a chromosomal factor.

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ТОМ XI

РЕЗЮМЕ

GENETIC RELATIONSHIPS BETWEEN MYCOBACTERIUM KANSASII AND MYCOBACTERIUM TUBERCULOSIS

J. Weissfeiler, V. Karassova, J. Holland

ГЕНЕТИЧЕСКИЕ СВЯЗИ MYCOBACTERIUM KANSASII С MYCOBACTERIUM TUBERCULOSIS

Й. Вейсфейлер, В. Карассова, Я. Голланд

Изучение биохимических, патогенных и серологических свойств двух штаммов *Mycobacterium kansasii* показало, что эти штаммы очень подобны тем штаммам *Mycobacterium tuberculosis*, которые резистентны к изониазиду и вирулентность их аттенуирована. В результате диссоциации удалось получить из обоих штаммов вариант „R". Вариант „R", полученный из штамма Polluk и Buhler, на основе его культуральных и иммунологических свойств, оказался аттенуированным штаммом *M. tuberculosis*. По мнению авторов, на основании этой находки, можно сделать вывод, что *M. kansasii* произошел путем мутации *M. tuberculosis* и, вследствие его патогенности для человека, его можно считать новым типом палочки туберкулеза.

SEROLOGICAL ACTIVITY OF MYCOBACTERIAL DESOXYRIBONUCLEIC ACID PREPARATIONS. I. EFFECT OF PURIFICATION AND HEATING ON THE COMPLEMENT FIXATION BY DESOXYRIBONUCLEIC ACID

I. Tárnok

СЕРОЛОГИЧЕСКАЯ АКТИВНОСТЬ ПРЕПАРАТОВ ДЕЗОКСИРИБОНУКЛЕИНОВОЙ КИСЛОТЫ, ИЗОЛИРОВАННЫХ ИЗ МИКОБАКТЕРИЙ. I. ВЛИЯНИЕ МЕТОДА ОЧИСТКИ И НАГРЕВАНИЯ НА РЕАКЦИЮ СВЯЗЫВАНИЯ КОМПЛЕМЕНТА ПРЕПАРАТОВ ДЕЗОКСИРИБОНУКЛЕИНОВОЙ КИСЛОТЫ

И. Тарнок

При изучении серологической активности препаратов дезоксирибонуклеиновой кислоты, изолированных из микобактерий и очищенных различными способами, установлено, что связывание комплемента присутствующими в качестве примеси другими веществами (белками, полисахаридами, возможно рибонуклеиновыми кислотами), можно устранить фенол-хлороформ-амилалкоголевым очищением. Комплементсвязывающую способность таким способом приготовленных, или же хроматографированных метилированным альбумином, препаратов можно снизить только путем деградации дезоксирибонуклеазы: карбоксипептидаза, рибонуклеаза, трипсин и лизоцим являются без эффективными.

При нагревании и быстром охлаждении препаратов дезоксирибонуклеиновой кислоты способность их связывать комплемент изменяется характерным температурным способом: активность при некоторых температурных величинах показывает максимум или минимум. Изменение активности оценивается как изменение «поверхности» антигена. При нагревании непосредственной связи между увеличением адсорбции, наблюдаемым в ультрафиолетовой части спектра (в 254 милимикрона), и изменением серологической активности не обнаруживалось.

В связи с исследованиями указывается на то, что присутствующие примеси в дезоксирибонуклеиновых кислотах, не выявляемые химическим и энзиматическим разложением, могут оказывать мешающее влияние.

EXAMINATION OF THE CYTOPATHIC EFFECT OF ADENOVIRUSES BY IMMUNOFLUORESCENCE

P. Geck, P. Dán, I. Nász

ИЗУЧЕНИЕ ЦИТОПАТОГЕННОГО ДЕЙСТВИЯ АДЕНОВИРУСОВ ИММУНОФЛЮОРЕСЦЕНТНЫМ МЕТОДОМ

П. Гекк, П. Дан, И. Нас

Изучены с помощью метода иммунофлюоресценции первичные клеточные культуры человеческого амниона, зараженные штаммами аденовируса 3 и 5. Выявлено, что морфологические образования, возникающие в ядре клетки на влияние вирусного заражения, обладают характерными для вируса антигенным свойством. Получены данные и в том отношении, что вирус, т. е. те части вируса, которые обладают специфическим антигенным свойством, вырабатываются уже 7—10 часов спустя после заражения. Можно было также определить, что образование вируса начинается не во всех клетках одновременно, все же в течение более длительного культивирования могли почти во всех клетках обнаружить возникновение специфической флюоресценции.

ENTERITIS ASSOCIATED WITH AN UNUSUAL BIOCHEMICAL VARIANT OF ESCHERICHIA COLI SEROTYPE O26 : B6 : H11

M. M. Ádám, T. Bartha, C. Kovách, B. Ringelhann

ЭНТЕРИТ, ВЫЗВАННЫЙ НЕОБЫЧНЫМ БИОХИМИЧЕСКИМ ВАРИАНТОМ ESCHERICHIA COLI СЕРОТИПА 026:B6:H11

М. М. Адам, Т. Барта, К. Ковач, Б. Рингельган

В яслях села Широк в январе 1963 г. появились групповые заболевания с энтеральными симптомами и явлениями со стороны дыхательных путей. Из кала больного грудного ребенка и здорового взрослого выделили разлагающий лактозу в течение 24 часов серотип 026:B6:H11 *E. coli*, а из кала двух больных грудных детей изолировали биохимический вариант того же серотипа, разлагающий лактозу в течение 6—10 дней. Штаммы, разлагающие лактозу в более поздний срок, по сравнению с предыдущими, дали хороший рост на дезоксихолат-цитратной среде и образовали колонии, подобные шигеллам. В сыворотке больных, за исключением одного, не получили оцениваемые титры агглютининов против выделенного возбудителя.

EFFECT OF CYSTEINE OF LOCAL SHWARTZMAN PHENOMENON

I. Jókay, A. Kiss, L. Kassai

ВЛИЯНИЕ ЦИСТЕИНА НА МЕСТНУЮ РЕАКЦИЮ ШВАРЦМАНА

И. Йокаи, А. Киш, Л. Кашшаи

У подготовленных для образования местной реакции Шварцмана кроликов, одновременно с разрешающей дозой эндотоксина, введенный внутривенно цистеин задерживает снижение числа тромбоцитов, возникающее под влиянием эндотоксина, тогда как снижение числа лейкоцитов в периферической крови, наоборот, усиливается под его влиянием. При даче цистеина у кроликов количественный феномен Шварцмана по продолжительности и интенсивности усиливается по сравнению с контрольными исследованиями.

NEW OBSERVATIONS ON HYDROGEN SULPHIDE PRODUCTION BY ENTERIC BACTERIA

K. Rauss, S. Vörös, T. Kontrohr

НОВЫЕ НАБЛЮДЕНИЯ В ОТНОШЕНИИ ОБРАЗОВАНИЯ H_2S ЭНТЕРАЛЬНЫМИ БАКТЕРИЯМИ

К. Раушш, Ш. Вёрёш, Т. Контрор

1. Энтеральные группы бактерий, несмотря на немногочисленные исключения, все образуют H_2S из цистеина или цистина. Метионин не является пригодным субстратом для образования H_2S.

2. На основе данных систематики, разницей между H_2S — положительными и отрицательными родами является то, что первые в состоянии образовывать H_2S и из сульфатов. Так, причиной «рутинной» продукции H_2S являются сульфатные примеси различных пептонов.

3. Добавление цистина к питательным средам с целью выявления образования H_2S не является выгодным, потому что при этом сглаживается дифференцирующий характер этой важной реакции, и она теряет свое значение.

4. Необходимо также избегать применения в качестве индикатора бумажной ленты, пропитанной уксуснокислым свинцом, обладающей чрезвычайной чувствительностью, с помощью которой выявляются также следы H_2S, лежащие ниже диагностического порога, и таким образом могут быть вызваны диагностические ошибки. Подходящим индикатором считается $(NH_4)_2Fe(SO_4)_2$ т. е. $FeCl_2$.

5. Для изучения образования H_2S изготовили стандартную питательную среду, содержащую пептон, лишенный сульфатов, в которой источником серы служит известное количество $Na_2S_2O_3$, а в качестве индикатора $(NH_4)_2$ Fe $(SO_4)_2$.

ALKALINE PHOSPHATASE ACTIVITY OF NORMAL AND POLIOVIRUS INFECTED HELA CELLS

I. Mécs, Gy. Domján, G. Szepessy

АКТИВНОСТЬ ЩЕЛОЧНОЙ ФОСФАТАЗЫ НОРМАЛЬНЫХ И ЗАРАЖЕННЫХ ПОЛИОВИРУСОМ КЛЕТОК HELA

И. Меч, Дь. Домьян, Г. Сепеши

Изучена гидролитическая активность щелочной фосфатазы нормальных и зараженных полиовирусом типа 3 (Saukett) клеточных культур HeLa. Установлено, что при стандартных условиях в нормальных клетках на активность энзима влияет возраст культур. В зараженных вирусом культурах, кроме того, на активность энзима влияет срок инкубации после заражения. В зараженных культурах максимум активности энзима наблюдался между 3—4 часами после заражения. Величина активности в клеточных культурах HeLa различного возраста была приблизительно одинаковая. Между тем способность этих культур к вирусообразованию с возрастом все больше снижается.

На основании этих явлений предполагается, что щелочная фосфатаза не принимает непосредственного участия в синтезе полиовируса, и что при заражении вирусом не возникает щелочной фосфатазный энзим нового типа. Это предположение подтверждается также константами Michaelis энзимов, и одновременно на основе этого можно исключить появление ингибиторов, влияющих на энзим в период „eclipse" синтеза полиовируса.

CANDIDA SOOSII N. SP., A NEW YEAST RELATED TO CANDIDA REQUINYII SZÉP ET NOVÁK 1963

E. K. Novák

CANDIDA SOOSII N. SP., НОВЫЙ ВИД ДРОЖЖЕЙ, РОДСТВЕННЫЙ CANDIDA REQUINYII (SZÉP ET NOVÁK 1963)

Е. К. Новак

Сообщает о штамме, изолированном как «дикие дрожжи» из реакторного загрязнения на дрожжевом заводе. Штамм считается новым видом, который в честь проф. д-ра Шоош Й. получил название *Candida soosii* и принадлежит к группе *Candida requinyii* рода *Candida*. *Candida soosii* n. sp. аскус не образует, вегетативное размножение его осуществляется путем почкования с псевдомицелиями (мицелий и артроспор не образует), крахмала и кератиноидного пигмента также не образует. Разлагает глюкозу и галактозу (слабо и удлиненно), может утилизировать, в качестве единственного углеродного источника, этанол (пленки не образует) и не разлагает арбутин.

PRODUCTION OF A LYTIC FACTOR BY ULTRAVIOLET LIGHT IRRADIATED CULTURES OF BACILLUS CEREUS. II. SOME PROPERTIES AND QUANTITATIVE DETERMINATION OF THE LYTIC FACTOR

S. Csuzi

ПРОДУКЦИЯ ЛИТИЧЕСКОГО ВЕЩЕСТВА КУЛЬТУРОЙ B. CEREUS, ОБЛУЧЕННОЙ УЛЬТРАФИОЛЕТОВЫМ СВЕТОМ II. КОЛИЧЕСТВЕННОЕ ОПРЕДЕЛЕНИЕ ЛИТИЧЕСКОГО ВЕЩЕСТВА И НЕКОТОРЫЕ ЕГО СВОЙСТВА

Ш. Чузи

Разрабатывался метод, пригодный для количественного определения литического вещества, образовывающегося при ультрафиолетовом облучении *B. cereus* 569. Изучен механизм литического действия этого вещества на культуру *B. cereus* 130.

При исследовании свойств литического вещества получены данные, доказывающие его энзимную природу. Также доказано, что адсорбция литического вещества на клетки *B. cereus* 130 является предпосылкой лизиса.

DIE STRAHLENEMPFINDLICHKEIT VON ESCHERICHIA COLI-KULTUREN I. PHYSIOLOGISCHE FAKTOREN VON EINFLUSS AUF DIE STRAHLENEMPFINDLICHKEIT

F. Hernádi, Gy. Csobán, Zs. Nagy

РАДИОЧУВСТВИТЕЛЬНОСТЬ КУЛЬТУР ESCHERICHIA COLI I. ФИЗИОЛОГИЧЕСКИЕ ФАКТОРЫ, ВЛИЯЮЩИЕ НА РАДИОЧУВСТВИТЕЛЬНОСТЬ

Ф. Гернади, Дь. Чобан, Ж. Надь

Изучена радиочувствительность культур *E. coli* в течение цикла роста. Установлено, что радиочувствительность в течение культивирования изменяется. Наибольшее изменение обнаруживается во время кинетически постоянной фазы „lag". Выявлено взаимное отношение между изменением радиочувствительности и формированием электродного потенциала культуры.

ADENOVIRUS HAEMAGGLUTINATION-INHIBITING ANTIBODIES IN HUMAN SERA

A. Lengyel, I. Nász

АДЕНОВИРУСНЫЕ АНТИТЕЛА, ЗАДЕРЖИВАЮЩИЕ ГЕМАГГЛЮТИНАЦИЮ, В ЧЕЛОВЕЧЕСКОЙ СЫВОРОТКЕ

А. Лендьел, И. Нас

Изучены антитела против типов 8, 9, 10, 11 и 16 аденовируса в сыворотках людей, перенесших кератоконьюнктивит. Среди 122 сывороток, взятых в период выздоровления, только у 10 не обнаруживался более высокий титр, чем 1:16 против аденовируса типа 8. У 42 больных исследовались пробы из обеих стадий заболевания (острой и фазы выздоровления). Против аденовируса типа 8 в остром периоде заболевания были отрицательными 80, а в период выздоровления только 5% сывороток. В течение болезни обнаруживалось повышение титра и против типов 9 и 10, тогда как против типа 11 этого не наблюдалось. Исследование сывороток 79 человек, ранее перенесших заболевание, показало, что задерживающие гемагглютинацию антитела выявляемы даже спустя 1 год после заболевания. Исследование 23 человек показало, что 4—6 месяцев после заражения титры являются более высокими, чем в сыворотках, взятых через 1 месяц.

В сыворотках 350 здоровых людей между встречаемостью антител против типов 8, 9, 10 и 13 аденовируса существенной разницы не было. Средняя положительность оказалась 42—47% у отдельных типов. В возрастной группе 15—45 летних частота по положительности была выше, чем у младших и старших возрастных групп. Положительность против типа 11 была низкая (15%), а против типа 16 гемагглютинацию задерживающих антител не обнаружили ни в крови перенесших кератоконьюнктивит, ни у здоровых людей.

DIE WIRKUNG VON TUBERKULIN AUF DIE ATMUNG VON ASCITESZELLEN IN VERSCHIEDENEN ZEITPUNKTEN NACH INFEKTION MIT MYCOBACTERIUM TUBERCULOSIS

O. Schweiger, B. B. Löw, A. Tomcsányi

ВЛИЯНИЕ ТУБЕРКУЛИНА НА ДЫХАНИЕ АСЦИТНЫХ КЛЕТОК МОРСКИХ СВИНОК В РАЗНОЕ ВРЕМЯ ПОСЛЕ ЗАРАЖЕНИЯ

О. Швейгер, Б. Б. Лёв, А. Томчаньи

Исследовалось дыхание клеток перитонеального экссудата морских свинок при наличии туберкулина в различные сроки заражения вирулентной палочкой у здоровых, вакцинированных БЦЖ, вакцинированных и десенсибилизированных, а также у леченных ГИНК и стрептомицином животных.

Дыхание клеток перитонеального экссудата морских свинок показало при наличии туберкулина только 24 часа после заражения усиленное дыхание, но 3, 14 или 42 дня спустя повышенного дыхания уже не обнаруживалось.

Дыхание клеток животных, иммунизированных БЦЖ за 6 недель до заражения — при наличии туберкулина — спустя 24 часа после заражения также усилилось, но это влияние в случаях не иммунизированных, зараженных животных через 3 дня после заражения не выявлялось, тогда как у иммунизированных животных оно наблюдалось и через 3 дня без изменения.

Дыхание клеток перитонеального экссудата морских свинок, которые иммунизировались, но десенсибилизировались туберкулином, при наличии туберкулина через 3 дня после заражения показало усиление, подобно тому, что обнаруживалось у иммунизированных, но не сенсибилизированных животных.

Дыхание клеток зараженных и леченных ГИНК животных при наличии туберкулина оказалось повышенным как через 24 часа, так и через 3 дня после заражения. Подобного явления не наблюдалось у животных, леченных стрептомицином.

ANTIGENIC STRUCTURE OF A NEW ENTEROPATHOGENIC E. COLI STRAIN

S. Vörös, B. Rédey, F. Csizmazia

АНТИГЕННАЯ СТРУКТУРА НОВОГО ЭНТЕРОПАТОГЕННОГО ШТАММА E. COLI

Ш. Вёрёш, Б. Редей, Ф. Чизмазия

На основе результатов исследования антигенной структуры авторами установлено, что гуманный энтеропатогенный штамм, изолированный в 1959 г. и сообщенный под названием *E. coli „Öskü"*, принадлежит к серогруппе *E. coli* 0.143, обладает до сих пор еще не точно определенным поверхностным антигеном характера B и содержит полный антиген *Sh. boydii* 8.

BREAKDOWN OF AMINO ACIDS BY ENTEROBACTERIACEAE. II. STEREOSPECIFICITY OF ALKALIZATION REACTION

B. Serény

РАЗЛОЖЕНИЕ АМИНОКИСЛОТ ЭНТЕРАЛЬНЫМИ БАКТЕРИЯМИ II. О СТЕРЕОСПЕЦИФИЧНОСТИ РЕАКЦИИ ПОДЩЕЛАЧИВАНИЯ

Б. Шерень

С помощью реакции подщелачивания у значительного числа энтеральных бактерий изучалась способность параллельного разложения формы laevo или dextro или racem отдельных аминокислот (альфа-аланин, серин, валин, триптофан, гистидин и глютамин). Различные сочетания бактерий-аминокислот дали разнообразные результаты. Иногда можно было установить стереоспецифичность у всех штаммов какой-то группы (подгруппы,типа) или же у большинства штаммов, другой раз только у меньшей части их. Обнаруживалось и полное отсутствие стереоспецифичности: всеми изученными бактериями группы (подгруппы, типа) разлагались все 3 формы. Поведение штаммов, принадлежащих к той же подгруппе или типу, в отношении стереоспецифичности может различаться между собой. В связи с выше указанным, на основании исследования некоторых штаммов нельзя сделать общепринятый вывод. Результаты реакции подщелачивания в растворе различных изомеров той же аминокислоты могут иметь диагностическое значение.

SIGNIFICANCE OF HAEMAGGLUTINATION-INHIBITING ANTIBODIES IN THE EVALUATION OF VACCINIAL REACTIONS

G. Nyerges, Gy. Losonczy, L. Erdős, Gy. Petrás

ЗНАЧЕНИЕ ГЕМАГГЛЮТИНАЦИЮ ЗАДЕРЖИВАЮЩИХ АНТИТЕЛ ПРИ ОЦЕНКЕ РЕАКЦИЙ РЕВАКЦИНАЦИИ

Г. Ньергеш, Дь. Лошонци, Л. Ердёш, Дь. Петраш

Исследовалась взаимосвязь между титром задерживания гемагглютинации до прививки и реакцией ревакцинации. Установлено, что лица, располагающие до прививки титром задерживания гемагглютинации $< 1:4$, имеют немного лучшие шансы в отношении эффективной ревакцинации, чем лица, у которых титр до вакцинации превышал 1:4. Все же до прививки не обнаруживалось такого титра, который делал бы эффективную ревакцинацию не вероятной. Неэффективные попытки ревакцинации нельзя было объяснить высоким содержанием в крови антител, задерживающих гемагглютинацию.

Эффективная вакцинация регулярно сопровождалась повышением в крови титра антител, задерживающих гемагглютинацию. Знание изменения титра антител, задерживающих гемагглютинацию, может способствовать правильной оценке сомнительных прививочных реакций.

FERMENTATION BY SYNCHRONOUSLY DIVIDING YEAST CELLS IN NITROGEN-POOR MEDIUM

T. Gánti

ИЗМЕНЕНИЕ СБРАЖИВАЮЩЕЙ СПОСОБНОСТИ СИНХРОННО ДЕЛЯЩИХСЯ ДРОЖЖЕВЫХ КЛЕТОК В СРЕДЕ БЕДНОЙ АЗОТОМ

Т. Ганти

Сбраживающая способность синхронно делящихся дрожжевых клеток в среде бедной азотом беспрерывно не увеличивается, а показывает периодически повторяющиеся возвраты, которые связаны с почкованием. Наблюдаемый эффект устраняется при добавлении анорганической соли азота. Величина эффекта имеет отношение к содержанию азота в дрожжах. Срок наступления возвратов зависит от температуры, при более высокой температуре наблюдается раньше. При росте интенсивности дыхания подобного эффекта наблюдать не представляется возможным. На основании выше указанных результатов автор предполагает, что размножающиеся в среде бедной азотом дрожжевые клетки могут из состава собственной сбраживающей энзимной системы временно выделять азот, если при их делении повышенная потребность в азоте не удовлетворяется из питательной среды.

STUDIES ON L FORMS OF STAPHYLOCOCCUS AUREUS STRAINS OF DIFFERENT ANTIBIOTIC AND PHAGE SENSITIVITY

M. Fodor, L. Miltényi

ИЗУЧЕНИЕ ФОРМ-L STAPHYLOCOCCUS AUREUS С РАЗЛИЧНОЙ ЧУВСТВИТЕЛЬНОСТЬЮ К АНТИБИОТИКАМ И С РАЗЛИЧНЫМ ВИДОМ ФАГА

М. Фодор, Л. Мильтени

У штаммов **Staphylococcus aureus** способность образовывать формы-L под влиянием 20 грамм/ml целбенина зависит от чувствительности к антибиотикам. Резистентные к антибиотикам штаммы менее способны образовывать формы-L, чем чувствительные. Штаммы с фаговым типом 80/81 и 52/52 A) 80/81 менее способны, чем другие штаммы, образовывать формы-L.

В жидкой питательной среде, содержащей 5% сахарозы, 10% человеческой сыворотки и 3% поваренной соли, среди 10 форм-L штаммов **Staphylococcus aureus** 8 пышно культивировались в форме слизистого осадка.

Человеческую сыворотку в питательной среде можно заменить сывороточными фракциями альфа-гамма-глобулина или альбумина в концентрации 0,3% или выше.

Среди 8 штаммов-L, хорошо культивировавшихся на жидкой сывороточной питательной среде, 2 дали рост также на полусинтетической среде, содержащей 0,3% **proteos pepton DIFCO**. Максимальный рост наблюдался через 18—24 часа. Возврат штаммов после прекращения дозировки целбенина не наблюдался.

На применяемых двух питательных средах наблюдалась сильно выраженная морфологическая разница у культур того же штамма.

ISOLATION AND CHARACTERIZATION OF SOME B. SUBTILIS PHAGES WITH "TRANSFORMING" ACTIVITY

J. Földes, J. Molnár

ИЗОЛИРОВАНИЕ И ХАРАКТЕРИСТИКА ФАГОВ B. SUBTILIS С ТРАНСФОРМИРУЮЩЕЙ СПОСОБНОСТЬЮ

Й. Фёльдеш, Й. Мольнар

Для цели получения трансформирующей нуклеиновой кислоты авторами выделены многочисленные фаги **B. subtilis**. Определены некоторые характерные черты (адсорбционная способность, период латенции, фаговая продукция одной клетки), и разгруппированы они с помощью серологических методов. Изолированные авторами фаги сопоставлены с фагами, изолированными другими авторами в последнее время, и на основании антигенной структуры между фагами, кажущимися темперированными, обнаружена более тесная связь. Нуклеиновая кислота, изолированная из вирулентных фагов, трансформировала компетентные клетки **B. subtilis**.

STUDIES ON THE HUMAN INTESTINAL FLORA. I. THE NORMAL INTESTINAL FLORA AND THE STABILITY OF ITS CONSTITUENTS

I. Kétyi, K. Barna

ИССЛЕДОВАНИЯ КИШЕЧНОЙ ФЛОРЫ ЧЕЛОВЕКА I. НАБЛЮДЕНИЯ В ОТНОШЕНИИ НОРМАЛЬНОЙ КИШЕЧНОЙ ФЛОРЫ И СТАБИЛЬНОСТИ ЕЕ ПОСТОЯННЫХ КОМПОНЕНТОВ

И. Кетьи, К. Барна

1. В материале авторов кишечная флора состояла из 4-х постоянных компонентов **E. coli, Str. faecalis, L. bifidus, Bacteroides**. Акцидентально встречались в последовательной их частоте следующие микроорганизмы: **Clostridia, Kl. pneumoniae, Staphylococci, Proteae**, микроаерофильные лактобациллы, **Saccharomycetales**, аэробные спорогенные и **Ps. aeruginosa**.

2. В отношении постоянных компонентов наивысшую относительную пропорцию представляла собой **E. coli**, а затем следовали **L. bifidus, Bacteroides, Str. faecalis**. Относительно высокая пропорция **E. coli**, достоверно высокое рассеивание аэробных компонентов и расхождение при их распределении, появляющееся главным образом в зоне более высокого числа бактерий, объясняется тем, что материал авторов сложился в первую очередь из более пожилых, постельных больных, где необходимо было считаться с расстройствами пассажа. Это подтверждалось и тем, что в старших возрастных группах имелись отклонения только в отношении более высокого числа бактерий **E. coli** и в пропорции сложившейся вследствие этого.

3. Число колоний, следовательно и соотношение постоянных компонентов кишечной флоры, показало индивидуальное отклонение высокой степени, тогда как у идентичного лица обнаруживалось далеко идущее постоянство числа бактерий.

4. Стабильность численности постоянных компонентов кишечной флоры восстанавливается, даже при глубоко нарушенном равновесии, в чрезвычайно короткий, недельный срок. Этот восстановительный процесс, при исследовании нормализации **E. coli**, влиял в направлении восстановления исходного штамма.

5. В связи с обсуждением эубиоза или дисбиоза на основании большого расхождения, проявляющегося в постоянных компонентах кишечной флоры, и разницы, появляющейся в составных частях акцидентальной флоры, рекомендуется большая осторожность. Установление нарушения кишечной флоры возможно только на основании количественного исследования предыдущей нормальной флоры.

STUDIES ON THE NUCLEIC ACID METABOLISM OF CHORIOALLANTOIC MEMBRANE CELLS AFTER INFLUENZA VIRUS INFECTION

K. Barb, G. J. Köteles, F. Antoni, Gy. Takátsy

ИЗУЧЕНИЕ НУКЛЕИНОВО-КИСЛОТНОГО ОБМЕНА В КЛЕТКАХ ХОРИОАЛЛАНТОИСНОЙ ОБОЛОЧКИ, ЗАРАЖЕННОЙ ВИРУСОМ ГРИППА

К. Барб, Т. Й. Кётелеш, Ф. Антони, Дь. Такачи

Параллельными вирусологическими и биохимическими исследованиями изучено с одной стороны размножение вируса гриппа A_2 (штамм **Singapore** 1/57) в клетках хориоаллантоисной оболочки куриного эмбриона, инкубированного в течение 12 дней, а с другой стороны изучено влияние вирусного заражения на нуклеиново-кислотный и белковый обмен клетки хозяина в первом цикле размножения вируса. Первый, вновь образованный вирусный компонент, растворяемый антиген, был выявлен спустя 180 минут после заражения. Гемагглютинирующая способность и инфективность появилась через 270 минут. Употребляя P^{32} для обозначения нуклеиновых кислот, начиная с 90-ой минуты после заражения, специфическая активность общей РНК-ы в зараженных клетках оказалась выше чем в контрольных. Из гомогенизата клеток с помощью 70%-ного фенола изготовлялись фракции РНК-ы „**phenol retained**" и „**phenol released**". Количество фракций „**phenol retained**" РНК-ы до 90-ой минуты после заражения чрезвычайно быстро возрастало. В этот срок ее количество было 10-кратно выше в зараженных клетках, чем в контрольных. Во фракции „**phenol released**" РНК-ты увеличение активности можно было выявить спустя 240 минут после заражения или одновременно с появлением инфективных вирионов. Уже спустя 45 минут после вирусного заражения можно было обнаружить усиленное строение лизина C^{14} в ядерные белки.

THE EFFECT OF DIFFERENT ANTICANCER AGENTS ON INDUCIBLE SYSTEMS OF BACILLUS MEGATERIUM

E. Marjai, G. Ivánovics

ВЛИЯНИЕ РАЗЛИЧНЫХ АНТИКАНЦЕРОГЕННЫХ АГЕНТОВ НА ИНДУЦИРУЕМЫЕ СИСТЕМЫ BACILLUS MEGATERIUM.

Е. Марьяи, Г. Иванович

Бактериоцин *Bacillus megaterium*, мегацин индуцируется ультрафиолетовым облучением. Следовательно, имеет сходство с большинством лизогенных систем. Проводилось исследование с целью выявления индуцирования мегациногенных бактерий, подобно лизогенным системам, с отдельными химикалиями. Проделано сравнительное изучение влияния 20 различных химикалий на мегациногенный и лизогенный штамм *B. megaterium*. Большая часть изученных химикалий является известными антиканцерогенными агентами. Можно было установить, что часть алкилирующих агентов, а также отдельные антиканцерогенные антибиотики (митомицин-С и кразинофиллин) индуцировали обе системы одинаково. Те, которые индуцировали лизогенную систему, были и эффективные в отношении индукции образования мегацина. Между эффективностью и химической структурой установить общедействительное правило было невозможно. Среди веществ с подобной структурой, так напр. этилвиминов, были как эффективные, так и неэффективные, что указывает на то, что алкилирующие агенты с антиканцерогенным действием не имеют полностью тождественную точку действия на клетки.

Данными наблюдениями подтверждается гипотеза, что за генетический контроль мегациногенеза отвечает в большей степени дефективный профаг.

MYCOLOGICAL EXAMINATION OF CLINICAL MATERIALS

I. Vitéz, E. K. Novák

МИКОЛОГИЧЕСКИЕ ИССЛЕДОВАНИЯ НА ОСНОВЕ КЛИНИЧЕСКОГО МАТЕРИАЛА

И. Витез, Е. К. Новак

Авторами на основе различного клинического материала выделены и идентифицированы 63 штамма грибов. Эти исследования дают дальнейшие возможности к распознаванию грибковой флоры больного человеческого организма. Обращается внимание на то, что при легочных процессах грибковые инфекции могут являться не только сопутствующими, но возможно, что и сами грибы способны вызывать в человеческом организме картину, подобную туберкулезу.

BIOCHEMICAL STUDIES ON STREPTOMYCES AUREOFACIENS IV. STUDIES ON THE BIOSYNTHESIS OF CHLORTETRACYCLINE

M. Járai, G. Józsa, J. Kollár

БИОХИМИЧЕСКОЕ ИЗУЧЕНИЕ STREPTOMYCES AUREOFACIENS IV. ИССЛЕДОВАНИЕ БИОСИНТЕЗА ХЛОРТЕТРАЦИКЛИНА

М. Ярай, Г. Ёжа, Й. Коллар

Применяя соединения, меченные C^{14} и Cl^{36}, при исследованиях установлено, что из органических соединений хлора к молекуле хлортетрациклина присоединяется атом хлора в присутствии хлорида. Это же наблюдали и у хлорпропандиола, который в штамме CDSD—314, образующем тетрациклин, не застраивается в скелет формулы тетрациклина, это значит, что он не является непосредственным прекурсором в биосинтезе хлортетрациклина.

Установлено, что из органических хлорных соединений атом хлора разлагается в одинаковой степени до хлорида двумя штаммами; штаммом B—28 и CDSD—314.

Подтверждено экспериментальным путем то предположение, что 2,5-**dimerkapto**-1—3—**4-tiadizoe** задерживает первую окислительную фазу биохимического хлорирования. В штамме CDSD—314, образующем тетрациклин, генетическая блокада биохимического хлорирования, вероятно, относится только к первой, окислительной фазе. В штамме CDSD—314 не найдена остаточная реакция при биосинтезе хлортетрациклина, следовательно, не обязательно генетическое биохимическое хлорирование двойным или несколькими альтернативными путями в период полной цепной реакции. Различное присоединение первого и второго атома С ацетатов может указывать на то, что второй атом С ацетата участвует как и единица C_1 в биосинтезе скелета формулы тетрациклина.

COMPARATIVE EXAMINATION OF CHRONIC TYPHOID CARRIERS WITH IMMUNOFLUORESCENT AND CULTURAL METHOD

P. Geck, R. Szántó

СРАВНИТЕЛЬНЫЕ ИССЛЕДОВАНИЯ МЕТОДАМИ ИММУНОФЛЮОРЕСЦЕНЦИИ И КУЛЬТИВИРОВАНИЯ У ТИФОЗНЫХ БАЦИЛЛОНОСИТЕЛЕЙ

П. Гекк, Р. Санто

Проверяли кал 200 зарегистрированных тифозных бациллоносителей методом иммунофлюоресценции и полученные результаты сравнивали с данными традиционного культивирования, дополненного обогащением. С помощью рутинного бактериологического культивирования в 84 случаях (47%), а методом иммунофлюоресценции в 139 случаях (69,5%) могли выявить *Salmonella typhi* в кале бациллоносителя. В двух случаях не удалось подтвердить результаты культивирования методом иммунофлюоресценции. Результаты культивирования совпали в 95% случаев с иммунофлюоресцентной положительностью, полученной в часовой срок из того же кала. С помощью подщелачивания кала смогли в большей степени повысить специфическое свечение *Salmonella typhi*.

STUDIES ON THE HUMAN INTESTINAL FLORA
II. ALTERATION IN THE INTESTINAL FLORA OF PATIENTS TREATED WITH ANTIBIOTICS

I. Kétyi, K. Barna

ИССЛЕДОВАНИЯ ЧЕЛОВЕЧЕСКОЙ КИШЕЧНОЙ ФЛОРЫ
II. ИЗМЕНЕНИЯ, ВОЗНИКАЮЩИЕ В КИШЕЧНОЙ ФЛОРЕ ЧЕЛОВЕКА ПОД ВЛИЯНИЕМ ЛЕЧЕНИЯ АНТИБИОТИКАМИ

И. Кетьи, К. Барна

1. В материале авторов самое слабое влияние на кишечную флору оказывал хлорамфеникол, особенно при парентеральном его применении. Хлортетрациклин действовал на кишечную флору сильнее, особенно на нормальные, анаэробные компоненты. Самым сильнодействующим, одинаково как на нормальные аэробные, так и анаэробные составные части флоры, оказался эритромицин.

2. При устранении отдельных нормальных компонентов или всей кишечной флоры, увеличение числа отдельных нормальных *Str. faecalis* или акцидентальных (**Clostridia Staphylococcus**) микроорганизмов встречалось от случая к случаю, но закономерность, связанная с изменением кишечной флоры, не наблюдалась.

3. Наличие ***Staphylococcus aureus*** в кале успокаивающим образом объяснялось подтверждением его происхождения из дыхательных путей.

4. Антибиотические инциденты в случаях авторов не наблюдались даже при тяжелых нарушениях или изменениях кишечной флоры. Поэтому в отношении антибиотических инцидентов или вторичных инфекций авторы заняли позицию, считая, что лишь, выпадение отдельных компонентов кишечной флоры или размножение отдельных микроорганизмов само по себе не является этиологическим фактором.

5. Экспериментальные опыты авторов на животных в связи с антагонистическим соотношением между отдельными компонентами кишечной флоры или же отсутствие последнего подтвердились наблюдениями, полученными в течение изменений кишечной флоры.

IMPLANTATION ANTAGONISM BETWEEN E. COLI AND NON-PATHOGENIC OR FACULTATIVELY PATHOGENIC ENTERIC BACTERIA

I. Kétyi

ИМПЛАНТАЦИОННЫЙ АНТАГОНИЗМ E. COLI ПРОТИВ АПАТОГЕННЫХ ИЛИ УСЛОВНО ПАТОГЕННЫХ РОДОВ КИШЕЧНОЙ ФЛОРЫ

И. Кетьи

1. Имплантационного антагонизма между родом **E. Coli** с одной стороны, а с другой трибом **Proteae** и родом **Klebsiella** не имеется. Внутри отдельных двух этих родов или трибов также не наблюдается антагонизм имплантационного механизма.

2. Антагонизм, обнаруженный между родами *E. coli* и **Klebsiella**, а также внутри рода **Klebsiella** отличается от характера имплантационного антагонизма.

3. У мышей зараженных перорально штаммами **Pseudomonas**, **Staphylococcus** не удалось осуществить имплантацию ни при наличии нормальной флоры, ни при отсутствии последней.

4. Имплантационного антагонизма или антагонизма другого характера и даже закономерной корреляции между *E. Coli* и нормальными постоянными компонентами *L. bifidus*, *Bacteroides*, *Str. faecalis* кишечной флоры не имеется.

5. На основании отсутствия имплантационного антагонизма, ограниченности выделения и из-за подтвержденной в последнем случае возможности механического удаления установлено, что между группами **Proteus** и **Klebsiella** с одной стороны, и кишечным эпителием с другой стороны тесной связи нет. Исходя из этого, автор считает, что триб **Proteus** и род **Klebsiella** занимают только акцидентальное микроэкологическое положение в кишечной флоре.

BREAKDOWN OF AMINO ACIDS BY ENTEROBACTERIACEAE III. CHARACTERISTIC COLOUR REACTIONS IN THE PROTEUS-PROVIDENCIA GROUP

B. Serény

РАЗЛОЖЕНИЕ АМИНОКИСЛОТ СЕМЕЙСТВОМ ENTEROBACTERIACEAE III. ХАРАКТЕРНЫЕ ЦВЕТНЫЕ РЕАКЦИИ В ГРУППЕ PROTEUS-PROVIDENCIA

Б. Шерень

Автором найдено, что бактерии, относящиеся к группе **Proteus-Providencia**, на определенной химической среде, содержащей **ferri-ammoniumcitrat**, разлагают среди аминокислот **L-tyrosin, L-tryptophan, L-histidin, L-lysin.** Разложение аминокислот проявляется в цветной реакции, появляющейся как при макротесте, так и при быстром или микротесте. Реакция характерна для группы, поэтому применима для идентификации. Простое техническое выполнение делает пригодной данную реакцию для рутинных целей.

STUDIES ON KLEBSIELLA INFECTIONS BY PHAGE DETECTION AND PHAGE TYPING

H. Milch, S. Deák

ИССЛЕДОВАНИЕ ИНФЕКЦИИ KLEBSIELLA С ПОМОЩЬЮ ФАГДИАГНОСТИКИ И ФАГТИПИЗАЦИИ

Х. Милх, Ш. Деак

С целью выявления возбудителя тяжелых энтеритов, встречающихся в отделении преждевременно рожденных, бактериологические исследования дополнялись фаговыми исследованиями. Согласно результатам, полученных авторами, выявление специфического фага имеет большое значение в установлении этиологического диагноза. Путем адаптации изолированных с диагностической целью фагов к штаммам **Klebsiella** различного происхождения удалось выработать типовую схему, которая является пригодной для эпидемиологических исследований. Изолированные фаги охарактеризованы на основании морфологии бляшек, термочувствительности, лизогенности и серологических свойств.

Штаммы, принадлежащие к различным группам **Klebsiella**, с помощью специфических фагов распределены на 5 фаговых групп и 13 типов.

Изучено распределение штаммов **Klebsiella** по фаговым типам в связи с внутрибольничной эпидемией.

Среди изученных 802 штаммов **Klebsiella** были типизированы 546 (68%). Штаммы, вызывающие внутрибольничные эпидемии, в 73%, а штаммы, изолированные при спорадических случаях, в 40% оказались фагочувствительными.

Изучена взаимосвязь между антигеном К и фаговым типом штаммов.

COMPARATIVE SEROLOGICAL STUDIES ON THE EFFECTIVENESS OF MONOVALENT LIVE POLIOVIRUS VACCINES GIVEN ALTERNATIVELY IN THE ORDER 2-3-1 and 1-3-2

F. Fornosi, I. Tálos

СЕРОЛОГИЧЕСКАЯ ЭФФЕКТИВНОСТЬ ПРИМЕНЕНИЯ ЖИВОЙ МОНОВАЛЕНТНОЙ ПОЛИОВАКЦИНЫ В ОЧЕРЕДНОСТИ ПО ТИПАМ 2—3—1 и 1—3—2

Ф. Форноши, И. Талош

В трех детских коллективах были иммунизированы живой полиовакциной 3—12 месячные грудные дети, ранее не иммунизированные. Схема иммунизации была в одном коллективе 1—3—2 (очередность типов), а в двух других 2—3—1, за исключением 12 детей, которые во время иммунизации третьим типом заболели ветряной оспой и вследствие этого получили третий тип последним (схема: 2—1—3). Средний возраст иммунизированных был в контрольной группе 6,5 месяцев, в группе 2—3—1 6,0 месяцев, а в группе 2—1—3 7,7 месяцев. Питание детей грудного возраста до вакцинации во всех трех группах было одинаковым. Другие энтеровирусы (не полиовирусы) выделены: в контрольной группе у 26%, а в исследованных группах у 25% грудных детей, при исследовании способности нейтрализации вируса в контрольной группе в 33% случаев, а в исследованных группах в 31% случаев получили отрицательный результат против всех трех типов полиовируса.

Иммунный ответ на 2 тип оказался одинакого хорошим на влияние вируса, введенного в начале (конверсионное отношение 20/20 = 100%) и к концу вакцинации (конверсионное отношение 11/11 = 100%).

Титры антител против типа 1 в некоторых случаях повысились и на влияние типа 2 или типов 2 и 3. Иммунный ответ на влияние 1 типа в начале вакцинации был слабее (конверсионное отношение 10/12 83%), чем к концу вакцинации по схеме 2—3—1 (конверсионное отношение 24/24 = 100%).

Конверсионное отношение на влияние типа 3 в контрольной группе было 75% (9/12), по схеме 2—3—1 68% (22/32). Конверсионное отношение тех, которые получили тип 3 последним, было 91% (10/11).

EXCRETION OF SV-40 VIRUS AFTER ORAL ADMINISTRATION OF CONTAMINATED POLIO VACCINE

L. B. Horváth, F. Fornosi

ВЫДЕЛЕНИЕ ВИРУСА SV—40 ПОСЛЕ ДАЧИ ЗАГРЯЗНЕННОЙ ПЕРОРАЛЬНОЙ ПОЛИОВАКЦИНЫ

Л. Б. Хорват, Ф. Форноши

Среди 35 детей 9—12 месячного возраста, первично вакцинированных, после дачи полиовакцины типа 1, загрязненной вирусом SV—40, 10 детей выделяли вирус SV—40. Титр антител полиовируса типа 1 после вакцинации был у детей, выделяющих вирус SV—40, ниже, чем у тех, из кала которых не удалось изолировать вирус SV—40.

LCM INFECTION OF MICE THYMECTOMIZED IN NEWBORN AGE

P. Földes, I. Szeri, Zs. Bános, P. Anderlik, M. Balázs

ЗАРАЖЕНИЕ ВИРУСОМ ЛИМФОЦИТАРНОГО ХОРИОМЕНИНГИТА МЫШЕЙ С УДАЛЕННОЙ В НОВОРОЖДЕННОМ ПЕРИОДЕ ЗОБНОЙ ЖЕЛЕЗОЙ

П. Фёльдеш, И. Сери, Ж. Банош, П. Андерлик, М. Балаж

Согласно исследованиям зарубежных авторов наблюдалось, что белые мыши, размножающиеся вне инцухта, с удаленной зобной железой в новорожденном периоде являются резистентными к заражению ЛХМ. Инфицированных мышей можно было разделить на 3 группы: 1) Мыши, которые одновременно с контрольной группой или 1—2 дневным опозданием погибли, при классических симптомах ЛХМ-та, с макроскопическими остатками зобной железы, 2) Позднее погибшие (с 19—30 дневной инкубацией), с признаками "**wasting disease**". 3) Оставшиеся в живых, из мозга и крови которых на 33—53 день после заражения могли изолировать вирус ЛХМ-та. На основании гистологических исследований (мозг, селезенка, зобная железа) и гематологических находок обсуждаются возможные причины позднего падежа мышей и иммунное состояние оставшихся в живых животных.

STUDIES ON THE INTRATYPIC VARIANTS OF ECHOVIRUSES II. THE ROLE OF CELL RECEPTORS IN THE SELECTION OF ECHOVIRUS VARIANTS

M. Simon

ИЗУЧЕНИЕ ВНУТРИТИПОВЫХ ВАРИАНТОВ ВИРУСА ECHO II. РОЛЬ КЛЕТОЧНЫХ РЕЦЕПТОРОВ ПРИ СЕЛЕКЦИИ ВАРИАНТОВ ВИРУСОВ ECHO

М. Шимон

Подобно другим авторам установлено, что гемагглютинирующие штаммы вируса ECHO теряют гемагглютинирующую способность в течение пассажей, проведенных на определенных человеческих стабильных клеточных штаммах. При этом на других экспериментах автор показал, что в случае исследованных гемагглютинирующих типов вируса ECHO (3, 6, 7, 11, 12, 13, 19), наблюдаются вирионы двоякого рода: гемагглютинирующие (H^+) и негемагглютинирующие (H^-). Настоящими исследованиями доказано, что гемагглютинирующие штаммы изменяются благодаря тому, что размножение вирионов H^+ на этих культурах селективно задерживается.

Установлено, что клетки клеточных штаммов человеческого происхождения и человеческие эритроциты содержат исключительно рецепторы, влияющие на варианты H^+, свойства которых (чувствительность к трипсину и формалину и резистентность к периодату) и условия связывания их с вирусом сходны. Подобные рецепторы выявить из первичных клеток **rhesus** обезьяньей почки и из клеток, происходящих из последних клеточных штаммов, не удалось.

Рецепторами задерживается гемагглютинация вариантов H^+. Влияние рецепторного вещества на размножение вируса изменяется соответственно тому, производится ли исследование на однородных (человеческих) или чужеродных (обезьяньей почки) для рецепторов клеточных культурах. На однородных клеточных культурах задерживается, тогда как на чужеродных стимулируется размножение вариантов H^+. Последнее влияние является по-видимому следствием улучшения условий пенетрации вируса. В случае вариантов H^-, рецептор не влияет на размножение вируса, ни в человеческих и ни в обезьяньих клеточных культурах.

Применением задерживающих гемагглютинацию рецепторов при изолировании H^+ вирусов ECHO, в первичных клеточных культурах обезьяньей почки, можно во много раз повысить шансы изолирования их.

EXAMINATION OF THE SUSCEPTIBILITY TO VIRUS INFECTION OF HETEROPLOID MONKEY KIDNEY CELL STRAINS

É. Csonka, P. Ruzicska

ИССЛЕДОВАНИЕ ВОСПРИИМЧИВОСТИ К ВИРУСНОЙ ИНФЕКЦИИ ГЕТЕРОПЛОИДНЫХ ШТАММОВЫХ КЛЕТОК ОБЕЗЬЯНЬЕЙ ПОЧКИ

Е. Чонка, П. Рузичка

Изучена восприимчивость к вирусной инфекции 7 гетероплоидных штаммовых клеточных культур обезьяньей почки, полученных в лаборатории авторов. Среди штаммов полиовируса дикие штаммы дали подобные, а аттенуированные более низкие титры на клеточных штаммовых культурах, чем на первичных клеточных культурах обезьяньей почки. Пассируемые клеточные культуры обезьяньей почки могут быть использованы для вирусодиагностических целей.

THE EPIDEMIOLOGICAL SITUATION IN HUNGARY IN INTERNATIONAL COMPARISON

K. Solt

ЭПИДЕМИОЛОГИЧЕСКОЕ ПОЛОЖЕНИЕ ВЕНГРИИ В МЕЖДУНАРОДНОМ СОПОСТАВЛЕНИИ

К. Шолт

Сравнивались данные заболеваемости, в отдельных случаях летальности и общей смертности при 9 острых инфекционных болезнях по некоторым европейским странам. При кори 10—100 кратная разница в заболеваемости между отдельными странами является следствием различного характера требований предписывающих правил извещения и различной дисциплины выполнения извещений о заболеваемости. Циклические колебания в соседних странах не совпадают.

Среди изучаемых стран, самая высокая заболеваемость дизентерией наблюдается в Венгрии, Англии и Валэсе. Сопоставление данных заболеваемости, летальности и общей смертности говорит о том, что в странах, сообщающих о своей низкой заболеваемости, фактические показатели количества сообщенных случаев превышают в 50—100 раз.

Заболеваемость эпидемическим гепатитом проявляет повышающуюся тенденцию во всех странах за исключением Дании и Финляндии, где это вполне допустимо, вследствие цикла переболеваемости, после которого заболеваемость всегда снижается. Сопоставленные данные заболеваемости и смертности и здесь показывают, что число заболеваний выше, превышая 20—80 кратно число извещений.

Значительное снижение заболеваемости дифтерией, коклюшем, столбняком и полиомиелитом в Венгрии является следствием обязательных возрастных прививок. Заболеваемость дифтерией по сравнению с уровнем 1950 г., 16 кратно снизилась в 1960 г. и ниже по показателям, чем в остальных странах Средней Европы. Профилактические прививки не прекратили циклические колебания заболеваемости коклюшем, но достаточно сильно ее снизили. После вакцинации живой вакциной против полиомиелита зарегистрировано в 1961 г. 7, в 1962 г. 1, в 1963 г. 4 случая заболеваемости. В результате прививок против столбняка заболеваемость им снизилась троекратно, а у полностью вакцинированной 1—19 возрастной группе даже десятикратно.

Заболеваемость брюшным тифом в Венгрии в начале 30 годов являлась в Европе наивысшей, но под влиянием массовых прививок, введенных в конце 30 годов, наблюдалось снижение ее. Под влиянием происшедших после 1945 г. общественных и экономических изменений, а также гигиенических мероприятий заболеваемость в настоящее время колеблется около $5^{0}/_{000}$, следовательно ее уровень ниже, чем в соседних странах. Заболеваемость паратифом в Венгрии относительно низкая. Общая заболеваемость брюшным тифом и паратифом в Венгрии ниже, чем в Финляндии и данные совпадают со Швецией.

TRANSFORMATION OF STREPTOMYCIN MARKERS IN ROUGH STRAINS OF RHIZOBIUM LUPINI

R. Balassa, M. Gábor

ТРАНСФОРМАЦИЯ МАРКЕРОВ СТРЕПТОМИЦИНА У ШТАММОВ RHIZOBIUM LUPINI

Р. Балашша, М. Габор

Авторами изучена трансформация стрептомицино-депенденции у штаммов:

1. Частота стрептомицино-депенденции является очень низкой: в случае «гетеролог» трансформации 0,0002%—0,000001%, в случае «гомолог» 0,024—0,0004%.
2. Маркеры резистентности и депенденции стрептомицина трансформируются как отдельно, так и совместно.
3. Тот же самый препарат ДРК (дезоксирибонуклеиновой кислоты) может передать стабильную и нестабильную депенденцию.
4. Степень трансформированной депенденции или резистентности стрептомицина различна в отдельных группах трансформантов. У части трансформантов степень депенденции или резистентности стрептомицина равняется степени депенденции или резистентности донора, а у другой части она ниже, чем у донора.
5. Среди колоний трансформантов те, которые имеют большие размеры, в каждом случае являются резистентными к стрептомицину, а которые имеют небольшие или средние размеры, могут быть резистентными и депендентными к стрептомицину.
6. На основании полученных данных предполагается, что у **Rhizobium** „situs" депенденция и резистентность к стрептомицину локализованы в одном комплексе.

THIN LAYER AND GAS CHROMATOGRAPHIC ANALYSIS OF SYPHILIS ANTIGENS

J. K. Makleit, A. Réthy, P. Richter

ТОНКОСЛОЙНЫЙ И ГАЗОХРОМАТОГРАФИЧЕСКИЙ АНАЛИЗ ЛЮЭТИЧЕСКИХ АНТИГЕНОВ С РАЗЛИЧНОЙ СЕРОЛОГИЧЕСКОЙ АКТИВНОСТЬЮ

Й. К. Маклейт, А. Рети, П. Рихтер

Исследовали фосфатидный состав и жирные кислоты люэтических антигенов различной серологической активности. Установлено, что люэтические штамм-антигены на тонкослойной хроматограмме содержат 6 компонентов.

Между антигенами, дающими или не дающими подходящие серологические реакции, на тонкослойной хроматограмме выявилось существенное расхождение только в отношении компонента № 6, который обнаруживается в последних в меньшем количестве. Серологическая реактивность не подходящих антигенов, обогащенных этим компонентом, немного усиливается; все таки при наличии его в более значительном количестве, реакция передвигается в аспецифическое направление. Приблизительно 50% антигенов состоит из жирных кислот, среди которых в серологической активности предположительно играют роль масляная, линолевая и стеариновая кислоты. Между составными частями жирных кислот у антигенов различной серологической активности выявляется существенная разница в количественной пропорции и в соотношении между собой жирных кислот, имеющих 18 углеродных атомов и располагающих различной степенью насыщенности. В случае подходящих антигенов соотношение масляная/линолевая кислота превышает 1500, а у не подходящих не достигает 1000.

Указывается на возможность применения тонкослойных и газохроматографических исследований при процессе изготовления антигенов.

ALKALINE PHOSPHATASE REPRESSION BY INORGANIC PHOSPHATE IN BACILLUS ANTHRACIS AND BACILLUS CEREUS

J. Lantos, G. Ivánovics

РЕПРЕССИЯ ЩЕЛОЧНОЙ ФОСФАТАЗЫ АНОРГАНИЧЕСКИМ ФОСФАТОМ В ШТАММАХ BACILLUS ANTHRACIS И BACILLUS CEREUS

Й. Лантош, Г. Иванович

Изучалось образование щелочной фосфатазы *B. anthracis* и *B. cereus* на полусинтетической питательной среде, хорошо определенной в отношении содержания фосфатов. Установлено, что оба species образуют щелочную фосфатазу в случае, если содержание анорганических фосфатов в питательной среде низкое. При высокой фосфатной концентрации образование энзима не выявляемо, потому что в случае обоих видов образование фосфатазы репрессируется присутствующим анорганическим фосфатом.

A RAPID DIAGNOSTIC METHOD FOR THE IDENTIFICATION OF ENTERIC PATHOGENS. FLUORESCENT MICROAGGLUTINATION

P. Geck

БЫСТРЫЙ ДИАГНОСТИЧЕСКИЙ МЕТОД ДЛЯ ИДЕНТИФИКАЦИИ ЭНТЕРАЛЬНЫХ ВОЗБУДИТЕЛЕЙ. ФЛЮОРЕСЦЕНТНАЯ МИКРОАГГЛЮТИНАЦИЯ

П. Гекк

Разработан быстрый диагностический метод с целью быстрой непосредственной идентификации энтеральных возбудителей из кала. Вначале из соответствующего разведения кала страдающих острым заболеванием проводится микроагглютинация на предметном стекле, которая окрашивается акридин-оранжевым, затем исследуется под флюоресцентным микроскопом. Флоккулообразные микроагглютинаты, в зависимости от числа микробов, флюоресцируют зеленым цветом, тогда как другие составные части кала имеют оранжевый оттенок. От случая к случаю появляющиеся другие образования, располагающие также зеленой автофлюоресценцией оценке не мешают, так как они и в контрольном препарате присутствуют и имеют аморфное строение.

Чувствительность и надежность метода проверялась методом культивирования, проведенном из тех же проб кала.

Среди госпитализированных энтеральных больных исследовано 500 человек, а также 500 заболевших в течение вспышки дизентерии. Из первой группы оказались положительными при культивировании 23,2% а с флюоресцентной микроагглютинацией 50,8%. У последней группы получен положительный результат с помощью культивирования в 33,3% случаев, а с помощью флюоресцентной микроагглютинацией в 59,1% случаев.

GROWTH OF VARICELLA VIRUS IN CONTINUOUS MONKEY KIDNEY AND HUMAN THYROID CELL CULTURES

L. Géder, E. Jeney, É. Gönczöl

РАЗМНОЖЕНИЕ ВИРУСА ВЕТРЯНОЙ ОСПЫ НА ПАССИРУЕМЫХ КЛЕТОЧНЫХ КУЛЬТУРАХ ОБЕЗЬЯНЬЕЙ ПОЧКИ И ЧЕЛОВЕЧЕСКОЙ ЩИТОВИДНОЙ ЖЕЛЕЗЫ

Л. Гедер, Э. Енеи, Е. Гёнцёль

Изучено размножение вируса ветряной оспы на культурах HeLa, и HEp.-2, Detroit—6, пассируемых культурах обезьяньей почки II/I/a и III/I, первичных культурах обезьяньей почки, человеческой щитовидной железы и также на культурах фибробластов эмбриона человека. Среди упомянутых культур оказались пригодными для размножения вируса ветряной оспы только пассируемые культуры обезьяньей почки III/I, первичные культуры обезьяньей почки, культуры человеческой щитовидной железы и эмбриональных фибробластов.

Обнаруживая клетки при помощи ультразвукового облучения в культурах развивающейся обезьяньей почки III/I и человеческой щитовидной железы, получили гомогенизаты невысокой степени инфективности. С меньшей степенью инфективности оказалась и центрифугированная питательная жидкость этих клеточных культур. Инфективность питательных жидкостей снижалась при центрифугировании со скоростью больше 1300, но она оставалась и после центрифугирования с 1200. При фильтровании на фильтре G—5 препараты этих клеток теряли свою заразительность.

COLUMN CHROMATOGRAPHY ON DEAE CELLULOSE OF HERPES SIMPLEX VIRUS AND CYTOMEGALOVIRUS

M. Koller, L. Géder, F. Lehel, É. Gönczöl, J. Kiss

ХРОМАТОГРАФИЧЕСКОЕ ИССЛЕДОВАНИЕ ВИРУСОВ HERPES SIMPLEX И CYTOMEGALIA НА ЦЕЛЛЮЛЕЗНОЙ КОЛОНКЕ ДЕАЕ

М. Коллер, Л. Гедер, Ф. Лехел, Е. Гёнцёль, Я. Кишш

Исследована элюция вирусов Herpes simplex и Cytomegalia на целлюлозной колонке ДЕАЕ, с фосфатными буфферами, содержащими хлористый натрий различной молярности. Вирус Herpes simplex элюировался фосфатным буффером, содержащим 0,6 и 0,9 М, а вирус Cytomegalia фосфатным буффером, содержащим 0,3 М хлористого натрия.

Преобладающая часть клеточного протеина элюировалась в фосфатном буффере, содержащем 0,3 М хлористого натрия, таким образом с помощью этого метода была возможна только частичная очистка вируса Herpes simplex.

Элюция комплемент-связывающей активности и инфективности у обоих вирусов во всех фракциях шла параллельно.

FATTY ACIDS IN ENTERIC BACTERIA

L. Váczi, A. Réthy, I. Rédai

ЖИРНЫЕ КИСЛОТЫ ЭНТЕРАЛЬНЫХ БАКТЕРИЙ

Л. Ваци, А. Рети, И. Редай

Определен жирно-кислотный спектр штаммов E. coli, S. typhimurium, Sh. flexneri, B. proteus. Установлено, что спектр различных жирных кислот содержит идентичные жирные кислоты, различные штаммы отличаются между собой только в количественных отношениях отдельных жирных кислот.

Главная масса жирных кислот состоит из жирных кислот, содержащих 16 углеродных атомов.

Более патогенные штаммы **Sh. flexneri** и **B. proteus** содержат в значительно больших количествах жирные кислоты с циклопропановым кольцом.

На основании данных исследований метод определения спектра жирных кислот является непригодным для отделения друг от друга близко родственных **species** бактерий.

STUDIES ON LIPIDS IN PSEUDOMONAS PYOCYANEA

L. Váczi, J. K. Makleit, A. Réthy, I. Rédai

ИЗУЧЕНИЕ ЛИПОИДОВ PSEUDOMONAS PYOCYANEA

Л. Ваци, Й. К. Маклейт, А. Рети, И. Редай

Изучен состав фосфатидов и жирных кислот штамма различного возраста. Сделаны следующие выводы:

1. ***Pseudomonas pyocyanea*** содержит 7,2—7,7% чистого общего липоида, 75—80% которого является фосфатидом.
2. Количество общего липоида сухих бактерий с течением времени растет.
3. Фосфатиды состоят, главным образом, из компонентов типа лецитина и кефалина.
4. Они содержат на тонкослойной хроматограмме 5 компонентов, имеющих в трех культурах различного возраста идентичные качества.
5. Культуры трех возрастов содержат одинаковое количество — 12 жирных кислот.
6. Основным компонентом является пальмитиновая кислота. Жирные кислоты, имеющие больше 16 атомов углерода, составляют 90% жирных кислот бактерии, тогда как жирные кислоты, имеющие атомов углерода меньше 16, только 10% их.
7. В более старых культурах увеличивается количество жирных кислот, содержащих циклопропановое кольцо с 19 атомами углерода.

ETUDE COMPARATIVE ENTRE LES SOUCHES BCG DE PARIS ET DE BUDAPEST

L. Lugosi, F.-M. Lévy

СРАВНИТЕЛЬНЫЕ ИССЛЕДОВАНИЯ ПАРИЖСКИХ И БУДАПЕШТСКИХ ШТАММОВ БЦЖ

Л. Лугоши, Ф.-М. Леви

В период 1954—1957 гг. после прививок БЦЖ вакциной, изготовленной из штамма, полученного в 1933 г. из Парижского Пастеровского института и с тех пор поддерживаемого в Будапеште, у вакцинированных обнаружилась сниженная и быстро гаснущая аллергия. Первоначально пытались усилить эффективность вакцины повышением ее концентрации. При сравнительных исследованиях на мышах и морских свинках выявлено, что «подштамм» БЦЖ, полученный в Будапеште, располагает по сравнению с Парижским «подштаммом», применяемым в настоящее время в институте, более низкой активностью. Поэтому в Венгрии, начиная с 1960 г., для изготовления вакцины БЦЖ, вместо ранее применяемого будапештского штамма, применяется вновь Парижский штамм БЦЖ.

HYPOTHERMIA AND HORSE SERUM ANAPHYLAXIS

T. Szilágyi, B. Csaba, L. Miltényi, L. Kassai

ГИПОТЕРМИЯ И АНАФИЛАКСИЯ ВЫЗВАННАЯ ЛОШАДИНОЙ СЫВОРОТКОЙ

Т. Силадьи, Б. Чаба, Л. Мильтени, Л. Кашшай

Морских свинок сенсибилизировали лошадиной сывороткой, шоковое состояние вызывали инъекциями различных фракций сыворотки. Установлено, что наиболее выраженным анафилактогенным действием обладает глобулин β. Также определено, что сенсибилизированные лошадиной сывороткой морские свинки при гипотермическом состоянии против фракций со слабым анафилактогенным действием десенсибилизируются, тогда как против сильных анафилактогенов не десенсибилизируются.

ATYPICAL MYCOBACTERIA IN MONKEYS

J. Weissfeiler, V. Karassova, J. Holland

АТИПИЧНЫЕ МИКОБАКТЕРИИ У ОБЕЗЬЯН

Й. Вейссфейлер, В. Карассова, Я. Голланд

Изучены штаммы микобактерий, выделенные от двух обезьян с положительной реакцией на туберкулин, вскоре после их импортирования в Европу.

Штамм М—2 оказался микобактерией туберкулеза с аттенуированной вирулентностью. Штамм Zo_1 пигмента не образует, по биохимическим, иммуногенным свойствам и антигенной структуре резко отличается от микобактерий турберкулеза. Штамм не относится к микобактериям типа Battey.

BIOCHEMICAL CHLORINATION INHERITANCE IN STREPTOMYCES AUREOFACIENS

M. Járai

НАСЛЕДСТВЕННОСТЬ БИОХИМИЧЕСКОЙ ХЛОРИНАЦИИ У STREOPTOMYCES AUREOFACIENS

М. Ярай

Изучалась наследственность хлоринации у **Streptomyces aureofaciens** с помощью геновой рекомбинации. Хлорирующая активность рекомбинантов, полученных совместным культивированием смешанных суспензий конидий, хлорирующих (ele^+) и не хлорирующих (ele^-) биохимических мутантов, оказалась в каждом случае высокой, 92—100% потенциала общего тетрациклина. На основании их хлорирующей активности рекомбинанты можно было распределить в две группы: путем геновой рекомбинации происходили штаммы „**klorid-scavenger**" (с 100%-ной хлорирующей активностью) и „**klorid non-scavenger**" с 92—98%-ной хлорирующей активностью.

Исследования, проведенные акридин-оранжевым указывали на то, что ген хлоринации имеет по-видимому хромосомное происхождение.

Применяемые комплементарные ауксотрофные пары на основании рекомбинационной частоты распределяются в 3 группы: первая группа с высокой рекомбинационной частотой ($2{,}03 \cdot 10^{-4}$), вторая группа с низкой рекомбинационной частотой ($0{,}015 \cdot 10^{-4}$), а третья группа несовместима. Явление можно объяснить предполагаемой половой полярностью у **Streptomyces.**

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